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MECHANISM OF BIOSYNTHESIS OF MYCELIAL GLUCOSAN FROM PENTOSES BY ASPERGILLUS NIGER[1]

C. F. VAN SUMERE[2] AND PING SHU

Abstract

The biosynthesis of mycelial glucosan from glucose-1-C^{14}, xylose-1-C^{14}, and ribose-1-C^{14} by *Aspergillus niger* was studied. The results indicate that the pentoses were converted to glucose via transketolase, transaldolase, and isomerase reactions, followed by polymerization to form mycelial glucosan. The difference in labeling of glucose from ribose as compared with xylose suggests that D-arabitol may be involved as an intermediate in ribose metabolism.

The synthesis of polysaccharides in nature does not always follow a single pattern. For instance, the formation of dextran and glycogen are accomplished by the action of transglucosidase on sucrose and phosphorylase on glucose-1-PO_4 respectively. On the other hand, xylan in the wheat plant is not a polymerization product of free pentose, but is derived from glucose via glucuronic acid (2). It was considered of interest to study the pathway of the reverse process, the synthesis of glucosan from pentoses. The mycelial polysaccharides formed by a number of filamentous fungi grown in pentose media were found to be largely glucosan (7), thus providing suitable materials for such an investigation.

This paper presents the results obtained when glucose-1-C^{14}, ribose-1-C^{14}, and xylose-1-C^{14} were used as carbon sources for the growth of *Aspergillus niger*.

Experimental

Preparation of Aspergillus niger Mycelia

Aspergillus niger PRL 72-4 was grown in three different media containing 3% glucose, xylose, and ribose respectively. The basal medium contained 1 g. potassium dihydrogen phosphate, 0.25 g. magnesium sulphate heptahydrate, 2.5 g. ammonium nitrate, 10.6 mg. ferric chloride hexahydrate, 6.2 mg. zinc sulphate monohydrate, and 100 ml. distilled water. The fermentations took place at 27° C. in closed shake flasks containing 100 ml. medium

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(5); these specially constructed flasks are equipped with an inner trough containing 50 ml. of a 4 *N* NaOH solution. The fermentations were discontinued when the oxygen utilization became negligible. An aliquot of the sodium hydroxide was taken to determine the total amount of carbon dioxide produced and its specific activity.

Isolation of Glucose from Mycelial Material

The fermented culture broth was filtered and the mycelium washed with distilled water until free from soluble carbohydrates. The wet mycelial cake was then refluxed for 3 hours in 150 ml. of 1 *N* sodium hydroxide. This treatment solubilized 64.6% of the protein and only 16.8% of the carbohydrate. The residue was recovered by filtration, washed with water, and dissolved in 20 ml. of 72% sulphuric acid. This was immediately diluted to 15% sulphuric acid, and refluxed overnight. The neutralized hydrolyzate was evaporated *in vacuo* to a very small volume (approximately 5 ml.), 20 ml. of ethanol were added, and the insoluble salts were removed by filtration. The desalting process was repeated and the filtrate then evaporated to a syrup. This was dissolved in 50 ml. distilled water and decolorized with active charcoal (Darco G 60). The resulting colorless solution was then deionized with Dowex 50 (H^+) and Amberlite IR-4B (OH^-). The glucose sample thus prepared contained only 0.1% of amino nitrogen and was shown by paper chromatography to be free of pentose.

Degradation of Glucose

Glucose was degraded by *Leuconostoc mesenteroides* as described by Altermatt *et al.* (1) and the radioactivity measured by the method of Buchanan and Nakao (4).

Results and Discussion

The specific activities of mycelial carbon, carbon dioxide, and mycelial glucose carbon derived from glucose-1-C^{14} were higher than those derived from pentoses (Table I). This indicates that the glucose is directly incorporated in the mycelial cell and that the decarboxylation step in the hexose monophosphate shunt is in active operation. Xylose-1-C^{14} gave higher mycelium and carbon dioxide yields than ribose, but its C_1 was not efficiently incorporated into the total cell material or cell glucosan. On the other hand,

TABLE I

DISTRIBUTION OF C^{14} IN FERMENTATION PRODUCTS

Substrate added	Sp. act. of sugar fed (mμc./mM. C)	Wt. mycelium (g./100 ml.)	Sp. act. of mycelial C (mμc./mM. C)	CO_2 produced (mM.)	Sp. act. of CO_2 (mμc./mM. C)	Sp. act. of glucose isolated (mμc./mM. C)
3 g. xylose	100	0.775	44.3	49.6	82.4	48.4
3 g. ribose	100	0.630	72.8	32.5	74.0	98.2
3 g. glucose	100	0.855	88.7	47.7	103.5	102.7

TABLE II

DISTRIBUTION OF C^{14} IN MYCELIAL GLUCOSE PRODUCED FROM VARIOUS SUGARS

Carbon source	C^{14} as % of total in glucose molecule					
	C_1	C_2	C_3	C_4	C_5	C_6
D-Xylose-1-C^{14}	50.2	9.91	26.33	6.88	2.98	3.68
D-Ribose-1-C^{14}	37.4	8.67	18.35	3.71	1.65	30.18
D-Glucose-1-C^{14}	91.16	2.81	2.29	1.05	0.97	1.67

ribose gave a poor mycelium yield, and its C_1 was more efficiently incorporated into the total cell material and cell glucosan. The difference between the metabolic pattern for ribose and xylose is striking.

The distribution of labeled carbon in mycelial glucose derived from glucose-1-C^{14}, ribose-1-C^{14}, and xylose-1-C^{14} is shown in Table II. The mycelial glucose formed from glucose-1-C^{14} had 91% of the activity in carbon 1 while the remaining carbons were uniformly low. In the glucose derived from xylose-1-C^{14}, about 50% and 25% of the labeled carbon were found in C_1 and C_3 respectively, while the activities of C_4, C_5, and C_6 were rather low. On the other hand, ribose-1-C^{14} gave a glucose with almost equal activity at C_1 and C_6, and a fairly strong activity at C_3. These results suggest that the mycelial glucosan is a product of direct polymerization of glucose, while xylose and ribose are first transformed into glucose before polymerization. This is similar to the synthesis of cellulose in the wheat plant (2).

The distribution of labeled carbon in the cellular glucose suggests that the hexose monophosphate shunt is involved in the transformation of xylose to glucose. A part of aldopentose-5-phosphate is first isomerized to a keto-pentose-5-phosphate, presumably xylulose-5-phosphate, and then via transketolase and transaldolase reactions is converted to hexose-6-phosphate.

The hexose formed from a pentose-1-C^{14} by the transketolase–transaldolase reactions (assuming a negligible amount of recycling) would have 67% of the total activity at C_1 and 33% at C_3. Extensive recycling would increase the activity of C_2 and accordingly reduce the activities at C_1 and C_3. None or little of the activity should be detected in the rest of the carbons. This was found to be true for xylose-1-C^{14}. However, a striking difference was exhibited in the conversion of ribose to glucose, since the C_6 was almost as active as C_1. An additional pathway to the one discussed for the conversion of xylose to glucose must therefore be operating in the case of ribose.

The distribution of C^{14} in the glucose may readily be explained by assuming that a part of the ribose-1-C^{14} follows a second pathway in which the pentose molecule is inverted by the formation of a pentitol with subsequent oxidation at C_5. That such a route is possible is demonstrated by the recent observation that xylulose was converted to arabitol by a resting cell suspension of an osmophilic yeast (3). Moreover, a cell-free preparation of uredospores

of wheat stem rust can reduce ribulose to arabitol in the presence of reduced diphosphopyridine nucleotide (6). Thus, it might be expected that D-ribose is first converted by an isomerase to D-ribulose, which is then reduced to D-arabitol. This is subsequently reoxidized to D-xylulose in which the labeling would be in the C_5 position. After phosphorylation, this keto-pentose is converted to glucose-6-phosphate by the transaldolase–transketolase reactions, as already described for xylose. If this pathway for the conversion or ribose were operating exclusively, labeling in the glucose derived from ribose-1-C^{14} would be 67% in C_6 and 33% in C_3. Since C_{14} is distributed almost equally between C_1 and C_6 both the direct pathway as followed by xylose and the indirect route just described may be operating.

Acknowledgment

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THE EFFECT OF MAMMARY TRAUMA ON SPAYING ATROPHY OF THE UTERUS IN THE RABBIT, GUINEA PIG, AND RAT[1]

E. O. HÖHN

Abstract

Uterine atrophy following spaying was significantly reduced in 22 rabbits in which six nipples were ligated at the time of ovariectomy, in comparison with 22 controls merely ovariectomized. A possible bias in these results due to variation of relative uterine weight with body weight was avoided, as was the possible effect of pseudopregnancy.

Sensitivity of uterine segments to acetylcholine was more frequent among the nipple-ligated than the control animals; but this sensitivity was not obviously associated with high individual uterine weights, and the group difference observed is of doubtful significance.

No obvious effect of nipple ligation on vaginal and mammary epithelial development was observed but the large uteri of some of the experimental animals showed myometrial and some endometrial development in the uterine horns.

India ink injections into four nipples per rabbit or insertion of six ligatures through the skin of the back did not significantly reduce postspaying uterine atrophy. In ovariectomized rats and guinea pigs, nipple ligation produced no effect on uterine weight in comparison with controls merely ovariectomized. In the rats, vaginal smears indicated an uninterupted anoestrus in animals of both groups, for 4 weeks following the operations.

The investigators who originally described the effect of nipple ligation in ovariectomized rabbits concluded that the mechanism underlying this effect was entirely humoral. Their experiments bearing on this point are regarded as inconclusive and it is suggested here that nipple trauma may influence one of the endocrines by a nervous mechanism, this endocrine in turn acting on accessory sex organs such as the uterus.

Mammary traumatization accompanied by ovariectomy has been reported to reduce the normal postspaying atrophy of the accessory sex organs in rabbits (Peeters *et al.* (4)). Traumatization consisted of passing a double thread under the skin near the base of the nipple, bringing it out along the long axis of the nipple, and tying the two ends together. Four weeks after ovariectomy and ligation, rabbits in which four nipples had been thus ligated showed a marked reduction of sex organ atrophy, in 8 out of 12 animals, when compared to controls simply ovariectomized. The reported findings comprised: greater weight of "internal genital apparatus" per unit body weight, greater spontaneous contractility and sensitivity to pitocin of uterine test segments, uterine and vaginal hyperaemia (absent in the controls), greater endometrial proliferation in the corpora uteri, some myometrial proliferation in the corpora and horns, and stronger development of nipples and milk ducts. Nipple ligation delayed for 5 weeks after ovariectomy was almost ineffective.

The effect was attributed to an oestrogen liberated from traumatized breast tissue or released in response to stress from the adrenal cortex. To test the first hypothesis, Peeters *et al.* (5) transplanted one nipple into the spleen, to obtain liver inactivation of possible local oestrogens, in several

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Contribution from the Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta. This work was supported by a grant from the University of Alberta Medical Research Fund.

ovariectomized rabbits. Splenic adhesions, making a circulatory liver bypass possible, rendered most of the experiments inconclusive. However, a reduction of spaying atrophy in some of these animals was obtained. This was interpreted by these authors as evidence that an unspecified chemical substance liberated from the transplanted nipple was responsible for the effects on the genital tract.

It was felt desirable to reinvestigate the basic phenomenon of reduction of spaying atrophy by nipple ligation, and to study its occurrence in other species. The rat seemed particularly useful since vaginal smears would provide a sensitive indication of possible oestrogen release induced by nipple ligation.

Experiments on Rabbits

(*a*) *Uterine Weight after Ovariectomy and after Ovariectomy with Nipple Ligation*

Peeters *et al.* (4) mention that some of their control animals were pseudopregnant at the time of the operation. Pseudopregnant animals have enlarged uteri at ovariectomy and may well have larger uteri even 4 weeks later. Unless such animals were equally represented in the control and nipple ligated series, this might account for an apparent reduction of spaying atrophy in one group. To exclude this variable, the procedure of Peeters *et al.* was repeated with the modification that, to magnify the effect if possible, six

TABLE I

EFFECT OF NIPPLE LIGATION ON RELATIVE UTERINE WEIGHT IN RABBITS, OBSERVED 4 WEEKS AFTER OVARIECTOMY AND NIPPLE LIGATION

Nipple ligated ovariectomized		Controls: Ovariectomized	
Body wt., g.	Uterine wt., mg./100 g. body weight	Body wt., g.	Uterine wt., mg./100 g. body weight
2245	29.3	2240	23.9
2253	24.2	2255	8.9
2268	35.9	2263	7.4
2310	27.7	2370	18.3
2327	29.4	2380	26.4
2375	27.6	2450	27.6
2375	39.1	2477	16.3
2475	62.0	2472	44.1
2525	62.7	2536	39.4
2550	75.2	2675	52.9
2583	26.7	2610	31.4
2650	62.3	2709	45.8
2770	11.9	2722	18.9
2793	32.3	2770	47.7
2810	34.6	2745	33.5
2884	22.3	2815	14.9
2940	111.8	2938	15.4
2990	51.3	2940	36.3
3070	119.0	3015	62.5
3280	64.0	3026	52.9
3280	65.8	3228	70.9
4140	26.0	3375	22.1
	Mean 47.3 (±6)		Mean 32.6 (±4.4)

instead of only four nipples on each animal were ligated with (single strand) cotton threads. Two groups of 22 animals each were used. No animal was pseudopregnant, as shown by examination of the ovaries at ovariectomy. The results for uterine weights are given in Table I. All uterine weights referred to in this paper were determined on the wet fixed organs after removal of excess moisture. It will be noted that in animals simply spayed, uterine weight, even when expressed as the ratio uterine/body weight, tends to increase with body weight (which is presumably related to age). Hence comparison of uterine weights may be misleading unless the two groups are uniform as regards body weights of the animals in each. The 44 animals used in Table I are therefore arranged in pairs of matching weights though the animals were not actually studied in pairs. Peeters *et al.* merely give the average body weight of their animals; a bias in their data due to unequal allocation of animals according to body weight among control and experimental animals may therefore have occurred. In the experiments reported in Table I, in which these factors have been eliminated, a significantly higher mean value for uterine weight/100 g. body weight was nevertheless found in the nipple-ligated group. Mean uterine weight of the controls was 32.6 mg./100 g. (S.D. of mean 4.4), for the nipple-ligated group it was 47.3 mg./100 g. body weight (S.D. of mean 6). This difference of mean values is significant, $t = 2.4$, $P = .02$.

(*b*) *Sensitivity of Uterine Muscle to Pitocin and Acetylcholine after Ovariectomy and Ovariectomy with Nipple Ligation*

Contractility of uterine muscle was tested on segments, suspended in 36 ml. aerated van Dyke's solution (2), in a muscle bath, at 39° C. Segments were tested from 10 rabbits ovariectomized 4 weeks previously and from 14 animals ovariectomized and nipple-ligated 4 weeks previously. Generally, only one segment from each animal was tested. Spontaneous contractions were virtually absent in all. The incidence of contractile responses of segments from rabbits of the two groups to addition of pitocin (up to 1 unit in 0.1 ml. saline), or, failing a response to this, of acetylcholine (ACh) 1 ml. 1/1000 solution, is summarized as follows:

	Nipple-ligated group		Control group	
	Pitocin	ACh	Pitocin	ACh
No. of segments showing contraction	2	8	1	1
No. of segments tested	14	12	10	7

Curiously, there was no obvious correlation between sensitivity to either agent and uterine weight. The results above show no marked difference in pitocin sensitivity in the two groups. ACh sensitivity is more frequent in segments from the experimental group but the difference is only of borderline

statistical significance (P about .08, the data was analyzed for the author by two independent methods by Professor E. S. Keeping, Department of Mathematics, University of Alberta).

(*c*) *Histological Appearances after Simple Ovariectomy and Ovariectomy with Nipple Ligation*

No obvious differences were found in sections of mammary tissue, endometrium of the corpora uteri, or vaginae of animals of the two groups. However, the uterine horns of the nipple-ligated rabbits which had enlarged uteri showed (*a*) endometrial changes similar to those obtained in an earlier study (3) with small doses of oestrone (2–3 μg.) given to previously ovariectomized rabbits and (*b*) greater development of muscle and connective tissue in the myometrial layer.

(*d*) *The Effect of India Ink Injection into the Nipple and of the Insertion of Ligatures in the Skin of the Back*

Ten (nonpseudopregnant) rabbits received an injection of 0.2 ml. India ink into four nipples at the time of ovariectomy. Four weeks later the mean uterine weight was 35.5 mg./100 g. compared to 31.4 mg./100 g. in 10 control animals of matching body weight which were only ovariectomized. This difference in uterine weights was not significant ($P = .4$).

Ten (nonpseudopregnant) ovariectomized animals had six ligatures placed in plucked areas of the skin of the back. Their mean uterine weight 4 weeks later was 38.2 mg./100 g. compared to 32.4 mg./100 g. in 13 controls of matching body weight, merely ovariectomized. The difference is not significant (P = approximately .5). The experiment indicates that irritation due to a superficial thread ligature in the mammary tissue is more effective than similar irritation elsewhere.

Experiments on Rats

(*a*) *Uterine Weights and Vaginal Smears*

Nineteen white Wistar rats, approximately 10 weeks old, were ovariectomized and 19 others, also ovariectomized, had six nipples ligated in each animal. Ligatures in the rat mammary glands disappeared after a few days, being presumably gnawed out by the animals. Once weekly, ligatures lost in this manner were replaced, to ensure continuity of mammary irritation for a 4-week period. Daily vaginal smears on animals of both groups were taken for 6 days preceding the operation and were continued after the operation on alternate days for 4 weeks. The animals were then killed. Vaginal smears taken after ovariectomy were of anoestrus type throughout, except in four animals which showed oestrus smears up to 3 days after ovariectomy. This latter effect was regarded as due to completion of cycles initiated before ovariectomy. Results from animals of approximately similar body weights were compared in the two groups. The mean uterine weight mg./100 g. body weight was 63.3 for the control ovariectomized rats (S.D. of mean 6.4)

and 65.9 (D.S. of mean 5.2) for animals with ligated nipples at the time of ovariectomy. This difference in relative uterine weights was not found to be significant ($P = .5$).

(*b*) *Histological Findings*

Sections of the nipple region of mammary glands and uterine horns were examined from the animals of both groups. Mammary sections showed ducts only. No difference in the sections of these two tissues from animals of the two groups was observed.

Experiments on Guinea Pigs

Eleven guinea pigs were ovariectomized and in 11 others ovariectomy was combined with ligation of both nipples in each animal. As with the rats, weekly re-ligation of nipples of some of the animals was found necessary. After 4 weeks, the animals of both groups were killed. Mean uterine weight, mg./100 g. body weight for the ovariectomized animals was 87.7 (S.D. of mean 15.3) and 73.5 (S.D. of mean 12.6) for ovariectomized and nipple-ligated animals. The slightly lower value of the nipple-ligated animals was not statistically significant ($P = .2$).

As nipple ligation apparently did not affect uterine weight, histological examinations were not carried out.

Discussion

In agreement with the findings of Peeters *et al.* (4), the results reported here indicate that the uterine atrophy which ordinarily follows bilateral ovariectomy in rabbits is reduced by simultaneous nipple ligation, as shown by greater (relative) uterine weight and some difference in incidence of sensitivity to acetylcholine. Spurious effects due to the use of pseudopregnant and nonpseudopregnant animals or due to differences in animal size were avoided. On the other hand, histological examination of tissues from the present series gave no obvious indication of reduced atrophy of the epithelial elements of the mammary gland, uterine corpora, or vagina in the nipple-ligated animals. The large uteri of some of the nipple-ligated animals showed myometrial and some endometrial development. The greater uterine weights of nipple-ligated animals is attributed mainly to an effect on the myometrium. The state of the myometrium and to some extent the endometrium resembles an oestrogen response. Uterine weights indicating obvious reduction of postspaying atrophy of this organ were obtained in only about 40% of the nipple-ligated animals. Nipple ligation thus seems to act in a haphazard manner; this was also noted by Peeters *et al.* (5).

Their conclusion that nipple ligation affects uterine atrophy entirely by a humoral mechanism seems debatable. If the nipples which, after transplantation into the spleen, reduced uterine atrophy were autogenous, the effect can be attributed to the gland from which the nipple was excised (and which was presumably sutured) as much as to the spleen transplant.

The effect may therefore be attributed to irritation of mammary nerves, which in turn leads to release of a humoral agent, cf. prolactin and pitocin release induced by suckling. Such a mechanism could explain the erratic effect of nipple ligation, since the ligature would only be effective if close to nerve trunks or endings.

Nipple stimulation by suckling can induce gonadotrophin release in rats. Selye and McKeown (6) were able to induce progestational uterine changes in adult females by allowing the young of other rats to suckle the experimental females. In the present experiments and those of Peeters *et al.* (4, 5) on ovariectomized animals, the humoral agent, if a gonadotrophin, must be presumed to have directly acted on the uterus or to have stimulated a release of adrenocortical gonadoids.

A nonspecific traumatic stress response is not a satisfactory explanation of the phenomenon for the following reasons: Control and nipple-ligated rabbits were both subjected to the major trauma of ovariectomy, skin ligatures were less effective than nipple ligatures, and formalin injections in rats induce adrenocortical enlargement but no uterine or vaginal stimulation in rats (Albert (1)). The failure to demonstrate an effect of nipple ligation in rats and guinea pigs may indicate a genuine species difference but it could also be due to species differences in the time course of the effect.

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AN EVALUATION OF TWO METHODS OF ALGESIMETRY IN HUMAN SUBJECTS[1]

JAMES G. FOULKS AND EDWIN E. DANIEL

With the technical assistance of GEORGE KENT

Abstract

The effect of analgesic drugs and placebos on experimental pain thresholds has been studied in 60 untrained and three trained human subjects. Muscle compression (cuff method) and compression of tissues overlying bone (aesthesiometer method) have been employed as pain-producing stimuli. The two methods manifest a comparable degree of reproducibility. The following coefficients of variation were obtained: (1) subject-to-subject, 80%; (2) day-to-day, 20%; (3) moment-to-moment, 10%.

The threshold elevations produced by analgesics were small and, in most cases, not significantly greater than those obtained with placebos. Responses to various agents with each method of measurement tended to be consistent from one time interval to the next on a given test occasion. There also was a positive correlation between the simultaneous results as measured by the two different procedures in the case of agents producing significant threshold elevations. However, there was no correlation between the responses of the subjects to placebos and to analgesic agents. The majority of subjects were inconsistent in their reactions to placebos. Neither exclusion of placebo reactors nor training of subjects enhanced the accuracy of threshold reproducibility or the ability to discriminate between agents. Neither method of measurement revealed threshold changes to analgesics which were large enough to detect practical differences in the potency of various agents or in the effects of different doses of the same agent.

Introduction

The methods currently available for the evaluation of analgesic drugs in the laboratory are far from satisfactory. Techniques which involve the use of experimental animals may fail to differentiate adequately between analgesia and more generalized forms of central depression. Any attempt to measure the onset and intensity of painful sensations necessarily involves the evaluation of subjective responses, and therefore requires the use of human subjects. The radiant heat method for determining human pain thresholds has been employed extensively in recent years. The threshold values obtained with this device have been reported to possess a high degree of reproducibility (25). However, the ability of this technique to reveal threshold elevations following the administration of analgesic agents (potent as well as mild) has been questioned repeatedly (1, 6, 8). The originators of this device have stressed the importance of employing highly trained subjects in experimental algesimetry (10). The subjects employed in such studies generally have been few in number. Consequently, it has not been feasible to relate dose to response in terms generally applicable to larger populations which usually manifest a high degree of individual variability in response to drugs.

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The comparability of the cutaneous sensation produced by radiant heat with various types of visceral and aching somatic pain which often are encountered clinically has been questioned. More recently, alternative methods of experimental pain threshold determination have been advanced. One of these, which utilizes compression of the muscle of the calf, has been offered as a technique suitable for application to untrained subjects (7). Another method involving the application of pressure to tissues overlying bone also has been introduced (11, 19). Since the thresholds can be measured by these methods with a single stimulus of continuously rising intensity, they can be determined more rapidly and conveniently than with the radiant heat procedure. The present study was undertaken for the purpose of evaluating these two algesimetric methods in both trained and untrained subjects.

In recent clinical studies the suggestion has been advanced that the identification and elimination of subjects who respond frequently to placebos may improve the evaluation of analgesic agents (2, 16). The possibility that this technique may be usefully applied to the study of experimental pain in the laboratory also has been investigated in the present study.

Methods

A. Threshold Measurement

The following devices were employed for the estimation of pain thresholds: (1) A sphygmomanometer cuff applied to the calf of the leg and inflated to produce muscle compression (cuff method). (2) An air-driven plunger placed so that its small plastic tip compresses the soft tissues of the forehead against the underlying bone (aesthesiometer method).

(1) *The cuff method.*—The procedure employed was substantially that described by Deneau, Waud, and Gowdey (7) with the inclusion of the refinements of apparatus suggested by those authors. The tests were conducted upon supine subjects lying on an ordinary cot with the leg relaxed in a position of external rotation. For each subject the position of the "hook-on" cuff was the same each time that tests were performed, being that which was found during an initial familiarization period to produce the lowest and most consistent threshold values.

(2) *The aesthesiometer method.*—The procedure employed was a modification of that described by Hardy *et al.* (11). A lucite disk, 1 cm. in diameter, was fitted to the tip of a metal piston rod, which was placed perpendicular to and approximately at the center of the forehead of the subject. The apparatus was locked in a bracket attached to the cot, and brought into place as the subject remained in the same position utilized for the cuff test.

Both devices were adapted to operate with compressed air delivered at 10 p.s.i. Air delivery was regulated by solenoid valves which were actuated by electrical switches and operated by "push-button" controls. The application of pressure was initiated by an "operator" who read and recorded the threshold values as measured by a mercury manometer. The mercury column

also was calibrated in grams for the aesthesiometer, in conformity with the data reported by other workers who have used this instrument (11, 19). To facilitate reading, the mercury column was fixed and supported at the threshold level upon closure of the switch operated by the subject, while the pressure applied to the pain producing device was simultaneously released. The rate of rise of pressure was *not* critical within the range employed (20–40 mm.Hg per second). The magnitude of pain thresholds was not influenced by repetition at intervals as short as 15 seconds.

B. Analgesic Agents, Subjects, and Test Schedules

The following agents were employed (1) acetyl salicylic acid (ASA) (600 and 1200 mg. doses),* (2) codeine (30 mg.), (3) meperidine (100 mg.), and (4) placebos composed of starch and lactose.

Male medical students were employed as subjects for these tests. Following a preliminary familiarization run, the 60 untrained subjects each participated in eight subsequent tests (spaced at 2-week intervals) during which a drug or placebo was administered.

Three subjects were trained by daily familiarization procedures for a period of 2 weeks. These subjects each participated in 48 subsequent tests (4 days a week for a period of 12 weeks) during which a salicylate or placebo was administered.

C. Testing Procedures

Each recorded threshold value is the average of three closely spaced successive determinations. On each occasion, initial (zero time) threshold determinations were obtained with each device, and these measurements were repeated at 30, 60, and 120 minute intervals following the ingestion of the test agent.

The evaluation of subjective responses in human subjects requires a carefully designed system of controls. We have adopted procedures which conform with the principles generally recognized in this field (3, 5, 6, 13, 14, 15, 24). The various tablets employed were indistinguishable in appearance and number on each test occasion, and were swallowed whole so as to eliminate taste as a factor. The procedure was "double blind", both subjects and test operators remaining ignorant of the nature of the agents being used.

The distribution of the various agents among the subjects was completely randomized (Latin square procedure for unisolated interactions (9)) so that approximately equal numbers of each test combination were employed during a given testing session, fulfilling the requirement for "concurrent comparison". In each series, tests were carried out at the same time of day.

The operators were required to ask no questions and to make no comment to the subjects with regard to the threshold values. If subjects volunteered the information that they felt that a particular threshold had been incorrectly

*A soluble effervescent preparation of acetyl salicylic acid (Disprin®) (and a corresponding placebo) were also used in these tests. Since this preparation did not differ significantly from ASA in its influence on pain thresholds, these data have been omitted.

measured, the value was discarded and the test repeated. Otherwise every value was accepted as measured, and the first three values obtained with each device were recorded and averaged regardless of the degree of their agreement. Visual and auditory distraction of subjects was minimized during testing, but no attempt was made to restrict activity during the intervals between tests.

At the time of the familiarization run, subjects were given instructions as to the criterion for threshold detection (transition of quality of sensation of pressure to that of distinct pain). An attempt was made to produce a confident and anxiety-free mood. The importance of an alert but neutral attitude was emphasized. The students were aware of the general nature of the test procedure and its significance from a scientific point of view. The subjects displayed a co-operative and positive motivation toward the test procedure.

Results

I. Reproducibility of the Pain Threshold and Sources of Variation

Initial (zero time) threshold determinations (each consisting of three successive readings) were obtained for each subject on each occasion of his participation in the testing procedure. These comprise a comparable series of tests in "normal" untreated subjects, and provide a useful basis for assessing the reproducibility and analyzing the variability of the pain threshold as determined by each of the two test devices.

(*a*) *Subject-to-subject Variation*

A wide variation in individual response was observed. The mean of the 1440 individual readings in untrained subjects was 281 mm.Hg for the cuff method and 1564 g. for the aesthesiometer method. The value of the mean thresholds for individual subjects ranged from 78–530 mm.Hg for the cuff method and from 300–3000 g. for the aesthesiometer method. These values were distributed as would be expected in a "normal" population.

The coefficient of variation between the 60 untrained subjects on any 1 day of testing was approximately 0.50 (0.44–0.52) for each method of measurement. The initial threshold levels obtained with the two pain-producing devices show a significant positive correlation with one another. The average pain threshold for each subject (8 days of testing) was classified as above or below the mean of all subjects for each technique of threshold estimation, and the quantal responses analyzed by the chi-square test for consistency. There is a significant tendency for subjects with higher thresholds with one method of measurement also to produce values above the average with the other device ($\chi^2 = 7.63$, $P = < 0.01$).

(*b*) *Day-to-day Variation*

The coefficient of day-to-day variation for both trained and untrained subjects was approximately 0.20 (0.18–0.21), a value which is significantly different from that for subject-to-subject variation ($P = < 0.001$). In 1250 observations on three trained subjects, Hardy *et al.* (11) reported a similar

TABLE I

EFFECT OF TRAINING ON MOMENT-TO-MOMENT VARIATION IN PAIN THRESHOLD

	Coefficient of variation	
	1st day of testing	8th day of testing
60 untrained subjects		
Cuff method	0.10	0.07
Aesthesiometer method	0.14	0.11
	1st 8 days of testing	last 8 days of testing
3 trained subjects		
Cuff method	0.14	0.08
Aesthesiometer method	0.17	0.14

range of day-to-day variation. When the mean value for all the untrained subjects was determined for each day in the testing sequence, there was a uniform tendency for the threshold values to increase progressively from the first to the 8th day of testing. This increase, which was seen with both test methods, led to an average threshold for all subjects which was 30–35% greater on the 8th day of testing as compared with the first. The tendency for increasing threshold with repetition of experience was equally evident in the three trained subjects when the mean values observed on the last 8 of the 48 days of testing were compared with those for the first 8 days.

(*c*) *Moment-to-moment Variation*

The reproducibility of the pain threshold determinations also can be analyzed in terms of the three successive readings taken at 15-second intervals for each threshold determination. The coefficient of moment-to-moment variation was similar for the two methods of measurement. For all 60 untrained subjects on any one day, and for each of the three trained subjects on all 48 test days, this coefficient ranged from 0.07 to 0.18. The results with the cuff method appeared to be slightly less variable than with the aesthesiometer in both groups of subjects. At best, training leads to relatively minor improvements in the "accuracy" of threshold detection (i.e. degree of agreement between successive readings as reflected in decreased moment-to-moment variation) after repeated test experiences (Table I).

II. Effect of Drugs on Pain Threshold

For each subject on each test day, the mean pain threshold determined at "zero" time, prior to the ingestion of the test agent, was assigned the value of 100%. The mean pain thresholds determined at various intervals following the ingestion of the test agents were recorded as percentage change (plus or minus) of the initial (control) threshold for each subject. The effect of the various agents at each subsequent test interval was then expressed as the mean of these threshold changes for all 60 subjects.* The values are

*This device has the effect of eliminating variance due to subject-to-subject and day-to- day variation in threshold. The data are analyzed statistically on the basis of the "null" hypoth esis as described by Snedecor (23).

summarized in Table II. Comparisons of each dose of each agent with its placebo and with other doses or agents at the same time interval after ingestion are tabulated in Table III.

(*a*) *Cuff Method*

All of the agents administered led to threshold elevations which at one or more time intervals after ingestion were significantly greater than the initial determination at zero time. These threshold elevations were uniformly of a small magnitude (maximum = 13% with meperidine at 120 minutes). Moreover none of the threshold elevations seen with codeine and ASA were significantly different from the simultaneous changes observed with the placebo, a result which is at variance with the observations of Deneau *et al.* (7). Only meperidine produced threshold elevations which were significantly greater than those observed with the placebo and with other agents tested, an effect which was definite only 120 minutes after ingestion of the agent (Table III).

TABLE II

EFFECT OF ANALGESIC AGENTS AND PLACEBOS ON MEAN PAIN THRESHOLDS

Agent	Time after administration (min.)	Cuff method			Aesthesiometer method		
		% change in mean threshold	S.E. of mean	*P**	% change in mean threshold	S.E. of mean	*P**
Placebo	30	1.6	1.6	NS	2.9	1.7	0.10
(*a*) Untrained	60	4.4	1.9	0.02	3.3	2.2	NS
subjects	120	3.0	1.9	0.10	4.1	2.2	0.07
(*b*) Trained	30	4.8	2.2	0.05	6.3	3.1	0.05
subjects	60	7.3	2.6	0.01	2.4	2.9	NS
	120	4.1	2.2	0.10	1.3	2.7	NS
ASA (600 mg.)	30	4.2	1.9	0.04	3.0	2.3	NS
(*a*) Untrained	60	4.9	1.9	0.01	4.6	2.6	0.08
subjects	120	3.4	2.0	0.10	3.3	2.8	NS
(*b*) Trained	30	5.2	2.2	0.02	5.2	2.8	0.10
subjects	60	3.8	2.5	NS	4.0	2.6	NS
	120	4.8	2.7	0.10	0.6	2.6	NS
ASA (1200 mg.)	30	2.5	1.7	NS	5.0	1.0	0.001
Untrained	60	5.0	2.1	0.02	8.8	2.2	0.001
subjects	120	4.7	2.1	0.03	6.5	2.3	0.01
Codeine (30 mg.)	30	2.0	1.9	NS	−2.4	2.1	NS
Untrained	60	7.4	2.3	0.001	2.2	2.0	NS
subjects	120	4.4	2.1	0.05	−2.2	2.2	NS
Meperidine (100 mg.)	30	3.0	2.1	NS	−0.2	2.3	NS
Untrained	60	11.1	2.9	0.001	6.2	2.4	0.02
subjects	120	12.9	3.0	0.001	9.3	2.8	0.001

*NS = not significant ($P = > 0.10$). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

TABLE III

COMPARISON OF THE MEAN THRESHOLD CHANGES PRODUCED BY VARIOUS ANALGESIC AGENTS AND PLACEBOS AT CORRESPONDING INTERVALS AFTER THEIR INGESTION

Agents compared	Time after administration (min.)	Cuff method			Aesthesiometer method		
		Diff. of means*	S.E. of diff.†	P‡	Diff. of means	S.E. of diff.†	P‡
(A) Trained subjects	30	−1.0	3.1	NS	−1.1	4.1	NS
(1) ASA (600 mg.) vs.	60	−2.5	3.5	NS	1.6	3.9	NS
placebo	120	0.1	3.5	NS	−0.7	3.8	NS
(B) Untrained subjects	30	3.1	2.4	NS	0.1	2.7	NS
(1) ASA (600 mg.) vs.	60	0.9	2.3	NS	1.3	3.0	NS
placebo	120	0.8	2.3	NS	−0.8	3.1	NS
(2) ASA (1200 mg.) vs.	30	0.9	2.4	NS	2.1	2.7	NS
placebo	60	0.6	3.0	NS	5.5	3.0	0.07
	120	1.7	2.7	NS	2.4	3.0	NS
(3) ASA (1200 mg.) vs.	30	2.2	2.5	NS	2.0	3.0	NS
ASA (600 mg.)	60	0.3	2.7	NS	4.2	3.3	NS
	120	−0.9	2.6	NS	3.2	3.3	NS
(4) Codeine vs. placebo	30	0.4	2.4	NS	−5.3	2.5	0.04
	60	3.0	2.9	NS	−1.1	2.7	NS
	120	1.4	2.6	NS	−6.3	3.0	0.04
(5) Meperidine vs. placebo	30	1.4	2.6	NS	−3.1	2.5	NS
	60	6.7	4.3	NS	2.9	3.3	NS
	120	9.9	3.2	<0.001	5.2	3.6	NS
(6) Meperidine vs.	30	−1.7	3.0	NS	−3.2	3.3	NS
ASA (600 mg.)	60	5.8	3.3	0.09	1.6	3.7	NS
	120	9.1	3.3	<0.01	6.0	4.0	NS
(7) Meperidine vs.	30	0.5	2.4	NS	−5.2	3.7	NS
ASA (1200 mg.)	60	6.1	3.5	0.09	−2.6	3.7	NS
	120	8.2	3.5	0.02	2.8	4.0	NS
(8) Meperidine vs. codeine	30	1.1	2.6	NS	2.2	3.1	NS
	60	3.7	3.6	NS	4.0	2.9	NS
	120	8.5	3.1	<0.01	11.5	3.9	0.01

*Minus value = threshold smaller in the case of the first of the two agents in the sequence of their listing.

†S.E. of difference calculated as $\sqrt{\frac{\Sigma(\text{dev.})^2}{N^2}}$ for the untrained subjects. Since each subject had a single trial with each agent, these could be paired for the tabulation of deviations for each subject.

S.E. of difference calculated as $\sqrt{(\text{S.E.})_1^2 + (\text{S.E.})_2^2}$ for the trained subjects (who had several trials with each agent and whose responses could not be paired).

‡NS = not significant ($P = >0.10$). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

(*b*) *Aesthesiometer Method*

In general, the elevation of pain thresholds above their initial values was less striking with the aesthesiometer technique, and a highly significant degree of elevation was achieved only in the case of meperidine and the larger dose of ASA. In contrast to the results obtained with the cuff method, the superiority of meperidine over the placebo and the other test agents was not statistically significant with the aesthesiometer technique.* Training did not improve the ability of subjects to differentiate between 600 mg. doses of ASA and placebos with either method of threshold estimation (Table IIIA).

*The effervescent soluble salicylate preparation produced threshold elevations which were not matched by their corresponding placebo in the case of untrained subjects using the aesthesiometer method, but this finding did not extend to the trained subjects.

(*c*) *Consistency of Responses*

The above results are all expressed in terms of the gross means for a number of tests. We have seen that even the statistically significant threshold changes are small (5–15% of the mean) as compared with the standard deviation of each series (7–25% of the mean) or the coefficient of moment-to-moment variation (7–18% of the mean). In every series of tests, including those with the largest mean threshold elevations, a considerable proportion (one-fourth to one-third) of the measured thresholds after ingestion of the placebo or analgesic agent were depressed rather than elevated, in comparison with the initial values. The consistency with which subjects responded to a given agent at different time intervals after its ingestion or at the same time with different methods of threshold measurement is a further statistical index of the validity of the data. The coefficient of correlation (23) was used to evaluate the consistency of these responses. There was a highly significant correlation between 60 minute and 120 minute responses for each agent studied, with both methods of measurement in both trained and untrained subjects (Table IVA). No significant correlation was found between the responses to the various agents tested (Table IVB), i.e. there was no detectable tendency for untrained subjects whose pain thresholds were elevated by one agent to also manifest positive responses to any other analgesic agent.† Furthermore there was no correlation between subjects responding with threshold elevations to placebos and to analgesic agents.

Simultaneous threshold changes in response to placebos or to the smaller dose of ASA did not show a significant correlation between the two different methods of estimation in either trained or untrained subjests (Table IVC). However, among untrained subjects, moderately significant correlations were found for the larger dose of ASA and the two narcotics.

(*d*) *Effect of Analgesic Agents on Pain Thresholds among Subjects not Responding to Placebos*

Beecher *et al.* (2), taking note of the fact that placebos are remarkably effective analgesic agents, have suggested that certain individuals are prone to respond to placebos. Such subjects would dilute the data when placebos are compared with drugs producing analgesia by pharmacological rather than psychological mechanisms. The identification and elimination of such "placebo reactors" should "sharpen the focus on the desired effect", i.e. placebo non-reactors should be a more satisfactory group for differentiating the effects of analgesic drugs. Clinical studies supporting this premise have been reported (2, 16).

The classification of placebo responses on a quantal basis permits an examination of the applicability of this procedure to the estimation of experimental pain thresholds among untrained subjects in the laboratory. This techique requires that an arbitrary level of response be selected for

†The responses to the effervescent salicylate preparation and meperidine did show a positive correlation of borderline statistical significance at both the 60 and 120 minute intervals in the untrained subjects, but only with the cuff method of measurement.

TABLE IV

CONSISTENCY OF RESPONSES TO PLACEBOS AND VARIOUS ANALGESIC AGENTS

A. Comparison of responses to a given agent at successive intervals after ingestion (60 and 120 minutes)

		Cuff method		Aesthesiometer method	
		r*	P†	r	P†
1. Untrained subjects:	placebo	0.616	<0.001	0.537	<0.001
	ASA 600 mg.	0.456	<0.001	0.678	<0.001
	ASA 1200 mg.	0.744	<0.001	0.690	<0.001
	meperidine	0.579	<0.001	0.698	<0.001
	codeine	0.824	<0.001	0.616	<0.001
2. Trained subjects:	placebo	0.592	<0.001	0.596	<0.001
	ASA 600 mg.	0.469	<0.001	0.543	<0.001

B. Comparison of responses to two different analgesic agents as tested with the same method of measurement and at the same interval (60 minutes) after ingestion (untrained subjects)

	Cuff method		Aesthesiometer method	
	r	P†	r	P†
Placebo vs. ASA 1200 mg.	−0.075	NS	0.127	NS
Placebo vs. meperidine	0.060	NS	−0.009	NS
Placebo vs. codeine	0.023	NS	0.129	NS
ASA 1200 mg. vs. meperidine	0.044	NS	−0.240	0.02
ASA 1200 mg. vs. codeine	−0.055	NS	−0.186	0.08
Meperidine vs. codeine	0.105	NS	0.181	0.09

C. Comparison of responses to a given agent with different methods of measurement at the same interval (60 minutes) after ingestion

		r	P†
1. Untrained subjects:	placebo	0.143	NS
	ASA 600 mg.	0.088	NS
	ASA 1200 mg.	0.262	<0.01
	meperidine	0.351	<0.001
	codeine	0.327	<0.001
2. Trained subjects:	placebo	0.077	NS
	ASA 600 mg.	0.197	NS

*r = coefficient of correlation = $\dfrac{(\Sigma xy/N)-\bar{x}\bar{y}}{\sigma_x \quad \sigma_y}$; $t = r\sqrt{\dfrac{n-2}{(1-r)^2}}$.

†NS = not significant (P = >0.10). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

TABLE V

EFFECT OF ANALGESIC AGENTS ON MEAN PAIN THRESHOLDS AMONG CONSISTENT PLACEBO NON-REACTORS (UNTRAINED SUBJECTS)

Agent	Time after administration	Cuff method (22 subjects) % change in threshold	S.E. of mean	P*	Aesthesiometer method (23 subjects) % change in threshold	S.E. of mean	P*
ASA (600 mg.)	60	1.1	2.9	NS	−0.6	3.1	NS
	120	1.2	3.1	NS	1.1	4.7	NS
ASA (1200 mg.)	60	6.9	3.9	NS	11.1	3.6	0.01
	120	2.3	2.7	NS	4.6	4.2	NS
Codeine	60	5.5	3.9	NS	−2.5	2.9	NS
	120	0.6	3.8	NS	−5.6	3.7	NS
Meperidine	60	8.0	3.6	0.06	9.9	3.6	0.01
	120	11.0	4.3	0.03	16.7	3.0	0.01

*NS = not significant (P = >0.10). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

dividing reactions into positive and negative categories. We have chosen a threshold elevation of 7% of the initial threshold value for this purpose, so that values within two to three standard errors of the initial mean threshold would be excluded from the group designated as positive reactors.* The group designated as placebo "non-reactor" has been confined to those subjects who failed to manifest a threshold elevation greater than 7% above the initial value at any of the three test intervals after placebo ingestion. Although there is a significant tendency for placebo (and other) quantal responses to be stable on a given test day (note correlation in Table IVA), at least half of the subjects were inconsistent in this respect when all three test intervals were considered, and not more than 10–20% of subjects had consistently positive responses to placebos at all three intervals. The trained subjects showed a similar degree of inconsistency in response to placebos on separate test occasions, a finding which agrees with the observations on clinical patients (20).

The threshold responses of the untrained "placebo non-reactors" at 60 and 120 minute intervals after the ingestion of the analgesic agents are assembled in Table V. The threshold elevations observed in this group are generally similar in magnitude to those obtained by analysis of the responses of the entire group of 60 subjects (see Table II). With the reduction in the number of subjects, the elevations of thresholds above the initial values attained

*The classification of responses on a quantal basis also permits their analysis by the technique of correlated proportions as described by Mosteller (22), and utilized by Beecher *et al.* (2). In this procedure, the relative analgesic potency of two agents is evaluated by determining whether or not the number of subjects responding positively to one agent but not the other is significantly greater than the number responding positively to the second agent but not to the first. In general, the results obtained with this procedure agree with the direct comparison of gross means, although usually with a lesser degree of statistical certainty.

statistical significance with both time intervals and methods of measurement only in the case of meperidine.

A comparison of the responses of placebo "non-reactors" and "reactors" to various analgesic agents is presented in Table VI. The threshold elevations manifested by placebo non-reactors are greater than those of placebo reactors in relatively few instances, and significantly so only as measured by the aesthesiometer 120 minutes after the ingestion of meperidine (17 out of 23 placebo non-reactors had positive responses to meperidine, but only 14 of the 37 placebo reactors manifested comparable threshold elevations).

The exclusion of placebo reactors apparently improves the detection of differences between the threshold elevating effects of various analgesics with the aesthesiometer method of measurement but not with the cuff method (Table VII, compare Table III). With the aesthesiometer, the accentuation of the superiority of meperidine over ASA and codeine among placebo non-reactors is sufficient to produce statistical significance for this difference in spite of the reduction in size in the group. Failure to obtain enhanced differences in threshold elevation among placebo non-reactors with the cuff method, along with reduction in group size, resulted in a loss of the statistical significance for the superiority of meperidine over the other agents studied which had been demonstrated with the entire group of 60 subjects. A reduction in the apparent difference in analgesic effect of two agents as a result of the exclusion of placebo reactors has been reported in a recent study in which the evaluation of clinical pain relief was employed (18).

TABLE VI

RESPONSES TO ANALGESIC AGENTS OF "CONSISTENT PLACEBO NON-REACTORS" AS COMPARED WITH THOSE OF SUBJECTS WITH ONE OR MORE PAIN THRESHOLD ELEVATIONS AFTER THE INGESTION OF PLACEBOS (UNTRAINED SUBJECTS)

Agent	Time after administration (min.)	Cuff method			Aesthesiometer method		
		Diff. of means*	S.E. of diff.†	P‡	Diff. of means*	S.E. of diff.†	P‡
ASA (600 mg.)	60	6.7	3.8	0.10	5.3	4.8	NS
	120	4.0	4.0	NS	3.5	5.8	NS
ASA (1200 mg.)	60	+3.0	5.3	NS	+2.5	4.4	NS
	120	3.8	4.0	NS	3.0	5.0	NS
Codeine	60	2.9	4.8	NS	7.5	3.8	0.06
	120	5.9	4.6	NS	5.5	4.6	NS
Meperidine	60	4.0	5.5	NS	+5.2	5.0	NS
	120	3.0	6.0	NS	+12.1	5.0	0.03

*+ = Higher mean threshold for placebo "non-reactors".

†S.E. of difference = $\sqrt{(S.E._1)^2 + (S.E._2)^2}$. Since responses in different groups of subjects are being compared, these cannot be paired.

‡NS = not significant (P = >0.10). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

TABLE VII

COMPARISON OF MEAN THRESHOLD CHANGES PRODUCED BY VARIOUS ANALGESIC AGENTS AMONG "CONSISTENT PLACEBO NON-REACTORS" (UNTRAINED SUBJECTS)

Agents compared	Time after administration (min.)	Cuff method			Aesthesiometer method		
		Diff. of means	S.E. of diff.*	P†	Diff. of means	S.E. of diff.*	P†
ASA (1200 mg.) vs. ASA (600 mg.)	60	5.8	5.0	NS	11.7	5.7	0.05
	120	1.1	4.2	NS	3.5	6.3	NS
Meperidine vs. ASA (600 mg.)	60	6.9	7.1	NS	10.5	4.5	0.02
	120	9.8	5.3	0.08	15.6	6.3	0.02
Meperidine vs. ASA (1200 mg.)	60	1.1	6.5	NS	−1.2	5.5	NS
	120	8.7	6.1	NS	12.1	6.0	0.05
Meperidine vs. codeine	60	2.5	5.1	NS	12.4	4.0	0.01
	120	10.4	5.4	0.08	22.3	6.2	0.001
Codeine vs. ASA (600 mg.)	60	4.4	5.2	NS	1.9	4.8	NS
	120	0.6	4.6	NS	6.7	6.9	NS
Codeine vs. ASA (1200 mg.)	60	1.4	5.9	NS	13.6	6.4	0.04
	120	1.7	4.5	NS	10.2	6.5	NS

*S.E. of difference $= \sqrt{\frac{\Sigma(\text{dev.})^2}{N^2}}$.

†NS = not significant ($P = >0.10$). It should be noted that the significance attached to probability values in the range of 0.01 to 0.10 is considerably reduced when such a large number of comparisons are made.

Discussion

We have not observed elevations of experimental pain thresholds of the magnitude reported by others who have employed these two methods of testing (7, 11). The large and highly significant threshold elevations (50–60%) described by Hardy *et al.* (11) using the aesthesiometer method were obtained following intravenous administration of codeine and meperidine. In the present series of tests failure of codeine to produce an appreciable analgesic effect following oral administration agrees with the report of Beecher *et al.* (2), who found oral codeine ineffective in the relief of clinical pain. Failure to obtain marked threshold elevations with ASA is not surprising in view of the modest analgesic effect elicited by meperidine in this series of tests.

Wolff and his co-workers have laid great emphasis upon the importance of training for reliable performance in pain threshold studies. Beecher (4) has criticized the use of trained subjects on the ground that training actually may enable subjects to distinguish between analgesics and placebos by virtue of the other recognizable pharmacological effects which analgesic drugs (particularly narcotics) commonly produce. Insofar as threshold variation and reproducibility by individual subjects is concerned, the performance of our untrained subjects with the aesthesiometer device appears to be comparable to that reported by Hardy *et al.* for trained subjects (11), the coefficient of variation

from test to test being approximately 20% in both series. Moreover, we have been unable to obtain a significant improvement in threshold reproducibility or in ability to differentiate placebos from moderate doses of ASA by using trained subjects.

The mean elevations in pain threshold produced by analgesics in the present study are small, particularly in comparison with the coefficient of variation of each series of threshold values (7–20%), and the coefficient of moment-to-moment variation (7–18%). In no case was the range of values with analgesic agents sharply separated from that for placebos. Overlapping was extensive, with many negative values for analgesic agents as well as placebos. The positive correlation between the responses obtained with these two different methods of measurement is confined to those agents which produced statistically significant alterations in threshold (Table IVC). The tests for statistical significance of differences between means and for consistency of responses (correlation coefficients) suggest that the threshold elevations which have been observed in the present studies may be valid in spite of their small magnitude. However, the practical value of tests of this type is compromised by the fact that agents known to differ markedly in their potency, insofar as the relief of severe pain is concerned, show so little difference in their ability to raise the threshold to experimentally induced pain.

The description of alterations of threshold as a percentage of the initial threshold value is a common procedure. Limitations of this form of expression have been discussed by Hardy *et al.* (11). These authors prefer to translate their data into terms of sensory units referred to as "just noticeable differences" in pain intensity (JND's). These units are said to vary considerably for different modalities of pain when expressed as a percentage of the initial threshold. With the aesthesiometer method, the JND was found to be about 25% of the initial threshold value. The practical importance which can be attached to a mean threshold elevation which is only a fraction of a just noticeable difference in pain intensity seems questionable, even if the alteration in threshold is highly significant from a statistical point of view.

The assumption that the ability of drugs to raise the threshold to pain is proportional to their ability to render intense pain less perceptible is not necessarily valid. For instance, the lack of such a correlation was noted by Hardy *et al.* (10, p. 367). The comparison of the effect of similar actions of agents of different potency is analogous to that of comparing the effect of different doses of the same agent. When biological end points (e.g. threshold) are employed for scaling the intensity of effects (see (21)) the comparison of dose and effect often fails to reveal a simple mathematical relationship. In the case of analgesics, part of this difficulty may be due to the action of drugs to modify the "response" to pain independently of their effect on the perception of its intensity. Considerations of this type have led Beecher and his co-workers to adopt clinical "pathological" pain as the most desirable situation for evaluating the relative efficacy of analgesic agents. Special

techniques have been developed which utilize the "average" pain of a group of patients, and use quantal responses to differentiate analgesic efficacy (3, 4, 8, 17). Relative potency is estimated by comparison with the response to a standard agent (morphine) which, together with placebos, is interspersed in the testing pattern (5). However, no procedure thus far developed permits a distinction as to the relative contribution of all of the various factors involved in the mechanism of pain relief (4, 12).

The prominent role which suggestion may play in studies of this type is evidenced by the frequency and degree of threshold elevation in response to placebos (5). The classification of placebo responses on a quantal basis and the exclusion of subjects with positive placebo reactions, as recommended by Beecher and his co-workers (2, 20) apparently improved the ability of untrained subjects to discriminate between the analgesic effects of meperidine and weaker analgesic drugs, although only with the aesthesiometer method of measurement (Table VII). However, the significance of this finding is dubious. The expected result of this procedure would be a decrease in the effect produced by the weaker analgesic agents owing to the elimination of placebo reactions. Actually, the enhanced superiority of meperidine among placebo non-reactors is more often the result of the greater mean threshold elevation produced by meperidine in this group of subjects (especially at the 120 minute interval) (Tables II, V). The aesthesiometer placebo reactors had unexpectedly low responses to meperidine, instead of the relatively high values which had been anticipated. Moreover, the test for consistency of responses showed no significant tendency for individuals with substantial threshold elevations following the ingestion of various analgesic agents to be the same subjects who also responded positively to placebo administration (Table IVB). Placebo non-reactors, as compared with the remaining untrained subjects, generally tend to be hyporeactors with analgesic agents, as noted in clinical investigations (2, 20, 22), although this was not a consistent finding, and little significant difference between the responses of these two groups of subjects emerged during the present study (Table VI).

This method of treating the data stems from the basic assumption that placebo reactors constitute a discrete category of recognizable subjects. The identification of such reactors would provide an attractive solution to the problem of suggestion in analyzing subjective responses. However, the validity of this assumption can be questioned on several grounds. Pain thresholds were determined more or less simultaneously by two different methods of measurement, but analysis of the responses to placebos in untrained subjects showed no tendency toward agreement between the results of the two methods (Table IVC).*

The majority of subjects are inconsistent in their responses to placebos. Lasagna *et al.* (20) have recognized the importance of multiple observations in order to increase the accuracy with which subjects may be classified on the

*An effervescent placebo was used as a control for the soluble salicylate preparation. There was no correlation between subjects showing positive responses to these two different types of placebos by either method of measurement.

basis of their placebo responses. It would appear that the spectrum of suggestibility is a broad one. The exclusion of consistent placebo reactors will eliminate a relatively small part of the diluting effect of placebo responses. Unless a large proportion (up to 60–70%) of subjects are screened out on the basis of multiple placebo tests, occasional placebo responders will continue to dilute data on analgesic effects to a substantial degree.

A part of these difficulties probably relates to the fact that pain sensations are graded, and the detection of arbitrarily chosen biological end points (e.g. threshold, or 50% relief) is difficult when subjective responses are involved. Many of our subjects, even those with extensive training, felt that the threshold end point, while sharp and readily recognizable on some occasions, was vague and elusive on others.

The methods employed in the present study may permit the demonstration of significantly perceptible elevations of experimental pain thresholds as a result of the administration of some agents ordinarily considered to possess analgesic properties. In general, however, it appears that threshold elevations of the magnitude of those reported here, while statistically significant in some instances, are too small to permit any conclusions as to the relative potency of various analgesic agents, or various doses of the same agent, and bear no necessary relation to clinical efficacy.

Summary and Conclusions

The effect of analgesics on the threshold of perception of pain has been studied in a group of 60 untrained subjects and in three trained subjects. Two different techniques for the experimental production of pain have been employed: (1) the cuff method (muscle compression), (2) the aesthesiometer method (compression of tissues against bone). The system of controls has included the use of the double blind procedure, concurrent comparison of agents and placebos, randomization of agents, and minimal distraction of subjects. Several statistical methods have been employed in analyzing the data.

A similar degree of threshold reproducibility was found for the two methods. Variation between subjects was large (coefficient of variation = 50%). Variation in threshold of a given subject was considerably less, both from one day to another and from moment to moment (coefficient of variation = 20% and 10% respectively). Administration of most of the agents studied, both analgesics and placebo, led to statistically significant elevations of the pain threshold at one or more of the intervals after their ingestion with both methods of measurement. These differences were small (5–15% of initial threshold values) and only meperidine with the cuff technique led to threshold elevations which were significantly greater than those following ingestion of placebos. The two methods gave comparable results insofar as the analysis of consistency of response was concerned. The response of individual subjects to a given agent (and with a given method of threshold estimation) tended to be stable from one time interval to the next. Moreover, there was a significant

positive correlation between the responses to a given agent as measured more or less simultaneously by the two different procedures, in the case of those agents (meperidine, codeine, 1200 mg. doses of ASA) which produced statistically significant elevations of the pain threshold.

The majority of subjects manifested inconsistent responses to placebos on different occasions. The analgesic responses of placebo reactors were generally similar to those of placebo non-reactors. The exclusion of placebo reactors enhanced the distinction between agents in only one instance, and reasons for doubting the validity of this observation have been cited. There was no correlation between the reactions of untrained subjects to placebos and to analgesic agents or between placebo responses measured simultaneously by the two different methods of measurement. Training of subjects did not appear to improve threshold reproducibility or the differentiation of placebos from analgesic agents. The magnitude of those differences which are statistically significant is too small to permit any general conclusions as to the relative potency of various analgesic agents, or of different doses of the same agent.

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DISTRIBUTION OF ADRENOCORTICOTROPHIC HORMONE IN THE PITUITARY GLAND[1]

G. J. Rochefort and M. Saffran

Abstract

The highly vascular, anteromedial zone of hog and beef pituitary glands, which is rich in basophilic cells, contains a concentration of ACTH from 4 to 13 times that found in the rest of the adenohypophysis. The concentration of ACTH can be correlated ($r = 0.96$) with the relative proportion of basophilic cells, supporting the view that the pituitary basophiles are the source of ACTH. The distribution of both basophiles and ACTH in the rat adenohypophysis is more uniform than in hog or beef glands. The posterior lobe of all species examined (rat, hog, beef, and man) contains small, but significant, amounts of ACTH.

The adrenocorticotrophic hormone (ACTH) has been isolated in pure form from the pituitary gland of several species (4, 21, 48) and its amino acid composition and sequence have been determined (21, 22, 49). In spite of the relatively large amount of work done recently on this hormone, the distribution and the cell of origin of ACTH in the pituitary gland are not well established. Fig. 1, A and B, depicts cross sections of the hog pituitary gland cut in two planes. The gland is divided into several regions discernible to the unaided eye: the pituitary stalk, the posterior lobe, the intermediate lobe, the anteromedial zone (reddish), the posterolateral zones (whitish), and the remainder (of an intermediate color), which forms the bulk of the gland. In 1923, Smith (42) observed that basophilic cells predominate in the anteromedial area, while the posterolateral areas are composed mainly of acidophilic cells. The bulk of the gland (intermediate zone) contains a mixture of these stainable cells and chromophobic cells that do not stain well with either acid or basic dyes.

Six trophic hormones have been extracted from the anterior lobe of the pituitary gland. Somatotrophin (STH) and prolactin (lactogenic hormone) are secreted by the acidophiles (3, 12, 38, 42), while thyrotrophin (TSH) and the two gonadotrophins (FSH and LH) are secreted by the basophiles (9, 10, 36). The chromophobic cells are believed to be inactive (34). Table I lists some of the attempts at correlating the cell type with the secretion of ACTH. Most of the approaches were necessarily indirect, but Smelser (41) assayed extracts of the predominantly basophilic or acidophilic areas of the beef pituitaries by the adrenal weight method of Moon (32) and found six to eight times as much ACTH per unit weight of basophilic tissue as in the acidophilic

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TABLE I

ATTEMPTS TO CORRELATE ACTH WITH CELL TYPE

Year	Investigators	Method
	Concluded that acidophile cells secrete ACTH:	
1938	Bailiff (1)	Cytology, rats
1938	Haymaker and Anderson (16)	Clinicopathological data
1944	Heinbecker and Rolf (17)	Cytology, dogs
1949	Finerty and Briseno-Castrejon (11)	Cytology, rats
1952	Herlant (18)	Assay of cellular components of gland
1953	Soulairac *et al.* (43)	Histology
1953	Desclaux *et al.* (8)	Implantation of parts of beef glands in rats; histology
1954	Barnett *et al.* (2)	Protein solubilities and bioassay
	Concluded that basophile cells secrete ACTH:	
1921–38	Shumaker and Firor (40) Crooke and Russell (6) Severinghaus (39)	Clinicopathological data
1944	Smelser (41)	Assays on separated parts of beef pituitaries
1948	D'Angelo *et al.* (7)	Cytology, guinea pigs
1948	Giroud and Martinet (13)	Implantation of parts of beef glands into rats; histology
1950–52	Tuchmann-Duplessis (45, 46)	Cytology and assays of rat pituitaries
1951	Marshall (23)	Immunochemical method
1954	Kallmann and Gordon (19)	Cytology
1956	Knigge (20)	Cytology and assays of rat pituitaries

area. The present paper describes the application of modern methods for the extraction and assay of ACTH to the mapping of the distribution of the hormone and the correlation of cell type with hormone content.

Recently, Mialhe-Voloss (24–27) claimed that the posterior lobe of the pituitary of several species contains significant amounts of ACTH. Her findings have been confirmed and extended.

Materials and Methods

Rat Pituitary Glands

Male rats, of the Sprague-Dawley strain, weighing 140 to 200 g., obtained from Canadian Breeding Laboratory, Montreal, were acclimatized for about 1 week in a constant-temperature animal room. The rats were anesthetized with intraperitoneal nembutal in the animal room and were transported to the laboratory. The heads were removed with a guillotine, the top of the skull removed with scissors, and the brain carefully lifted out, exposing the hypophysis. The gland was freed from adhering tissues with fine forceps, the posterior lobe was carefully lifted off and, if saved for assay, placed in ice-cold Krebs–Ringer–bicarbonate medium (47) containing 200 mg.% glucose. Four to ten posterior lobes were pooled, drained on a dry glass plate, rapidly weighed on a micro torsion balance, and extracted by the modified glacial acetic acid method of Birmingham *et al.* (5); the extracts were assayed for ACTH by the *in vitro* method of Saffran and Schally (37).

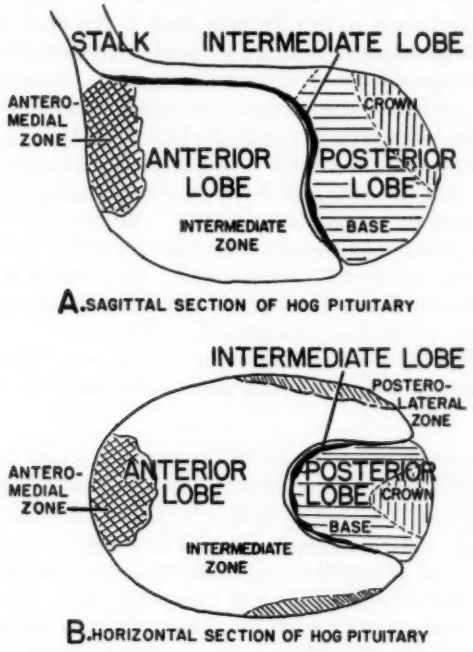

FIG. 1. Diagrammatic representation of the zones in cross sections of the hog pituitary gland. A. Sagittal section. B. Horizontal section.

The anterior lobe was halved *in situ* and both halves frozen on dry ice, either as removed from the animal or in a drop of distilled water. Each half gland was oriented on a freezing microtome and sectioned serially at 50 μ. Four consecutive slices from the right half of each of three glands were placed immediately into small centrifuge tubes (7 × 60 mm.), containing 100 μl. of glacial acetic acid; the corresponding slices from the left halves were placed on small tared paper disks. The tissue in the tubes was ground with fine sand and a small glass rod, an additional 50 μl. of glacial acetic acid was added, and the tubes were heated in a water bath kept at 80° C. After 30 minutes, the tubes were removed and the glass rods were washed with 50 μl. of glacial acetic acid. The tubes were sealed with Parafilm and stored in the deep freeze until their contents were assayed. The tissue on the disks was dried at 110° and then weighed on an ultramicro torsion balance to obtain dry weights of the sections. The fresh weight of rat anterior pituitary tissue is approximately five times the dry weight (44).

This procedure was repeated for each of three planes of the anterior lobe: sagittal, horizontal, and coronal.

Beef and Hog Pituitaries

The beef and hog pituitaries were obtained at the slaughterhouse as soon as possible after death of the animal, wrapped individually in aluminum foil

or Parafilm, quick frozen on dry ice, and kept in the deep freeze until used. Each gland was individually thawed and freed of adhering membranes. The posterior lobe was gently detached from the anterior lobe and, when used for assay, cut into two parts: the *crown* of the gland, containing the tissue most remote from the anterior pituitary, and the *base*, containing the tissue adjacent to the cleft and the anterior pituitary (Fig. 1). These separated tissues were rapidly weighed and dropped into 2-ml. volumetric tubes containing 500 μl. of glacial acetic acid for extraction and assay. With scalpel and fine scissors, the posterolateral zones and the anteromedial zone of the anterior lobe (Fig. 1) were dissected out, extracted, and assayed. In a series of hog glands, in addition to these two zones, a sample of the intermediate zone (Fig. 1) was also dissected out, weighed, and extracted.

One human pituitary, received frozen on dry ice, was thawed, and samples of the anteromedial and posterolateral zones were taken for analysis.

Histology

Specimens from the zones taken for assay were fixed in Lillie's sodium acetate – mercuric chloride – formalin or in Zenker–formol, embedded in paraffin, sectioned at 6 μ, and stained with the PAS – orange G technique (33). The nuclei were counterstained with Weigert's hematoxylin. The relative abundance of acidophiles and basophiles was determined by counting the nuclei contained in acidophilic and basophilic cells. Five random fields in each section were counted, each field being counted three times for each cell type. The field was about 16 μ square. The standard error of the count was calculated by the analysis of variance.

Results

Distribution of ACTH in the Rat Anterior Pituitary

The distribution of ACTH in the rat anterior pituitary is summarized in Tables II, III, and IV and Fig. 2. The zone of highest concentration of ACTH is located in the central horizontal plane in an area remote from the posterior lobe. This zone contains about twice as much ACTH as the rest of the anterior lobe, but the difference is not statistically significant.

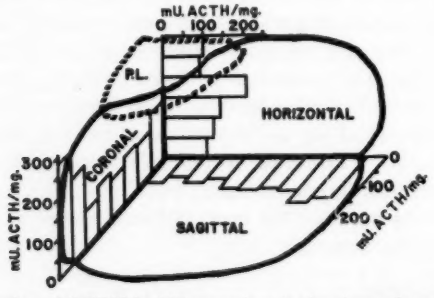

FIG. 2. Distribution of ACTH in 200-μ sections in the three planes of the right half of the anterior lobe of the rat pituitary gland. The area bounded by the dashed line, and labelled P.L., indicates the position of the posterior lobe.

TABLE II

ACTH IN SAGITTAL SECTIONS OF RAT ANTERIOR PITUITARY

Section	mU. ACTH/mg. of fresh tissue	95% limits of confidence	Index of precision
1	No detectable activity	—	—
2	135	79–231	0.12
3	151	58–393	0.18
4	108	59–198	0.10
5	102	54–193	0.11
6	106	83–135	0.03
7	101	44–231	0.15
8	71	21–245	0.16
9	84	39–178	0.15
10	63	36–110	0.10
11	84	52–170	0.12

TABLE III

ACTH IN HORIZONTAL SECTIONS OF RAT ANTERIOR PITUITARY

Section	mU. ACTH/mg. of fresh tissue	95% limits of confidence	Index of precision
1	101	56–181	0.12
2	80	46–136	0.11
3	207	104–410	0.09
4	142	105–192	0.05
5	127	87–183	0.08
6	107	65–177	0.09

TABLE IV

ACTH IN CORONAL SECTIONS OF RAT ANTERIOR PITUITARY

Section	mU. ACTH/mg.	95% limits of confidence	Index of precision
1	145	106–199	0.05
2	107	57–197	0.12
3	114	63–207	0.12
4	97	60–156	0.10
5	115	43–310	0.22
6	93	52–164	0.11
7	209	132–330	0.09
8	284	143–565	0.12

Distribution of ACTH in Beef, Hog, and Human Anterior Pituitaries

The anteromedial zone of beef anterior pituitaries contains 4 to 13 times as much ACTH as the posterolateral zones (Table V). These differences are statistically significant except in the case of gland No. 3.

As in the beef glands, the anteromedial zone of the hog pituitary gland has 4 to 10 times as much ACTH as the posterolateral or intermediate zones

(Table VI). Cell counts were made on PAS – orange G-stained sections of the assayed tissue zones of glands 4, 5, and 6 (Table VII). In Fig. 3, the percentage of basophiles in each section is plotted against the ACTH content of each tissue area. The correlation coefficient between the abundance of basophiles and the ACTH concentration is 0.96.

The human pituitary came from a 63-year-old male with no known endocrine disorder or medication, who died from bronchopneumonia following a transurethral prostatectomy. The posterolateral zone assayed at 113.6 mU. of ACTH per mg. of fresh tissue, with limits of 70 and 185, while the anteromedial zone assayed at 245 mU. of ACTH per mg., with limits of 169 to 355; both assays had an index of precision of 0.08. While this difference is not significant, again the anteromedial zone tended to be richer in ACTH.

TABLE V

ACTH IN BEEF ANTERIOR PITUITARY

Gland	Tissue zone	mU. ACTH/mg.	95% limits	Index of precision
1	Posterolateral	21.0	10– 42	0.14
	Anteromedial	152.0	62–370	0.19
2	Posterolateral	14.0	5– 41	0.20
	Anteromedial	183.0	84–397	0.17
3	Posterolateral	15.5	9– 27	0.10
	Anteromedial	133.0	18–941	0.22
4	Posterolateral	35.0	21– 58	0.08
	Anteromedial	140.0	98–200	0.08

TABLE VI

ACTH IN HOG ANTERIOR PITUITARY

Gland	Tissue zone	mU. ACTH/mg.	95% limits	Index of precision
1	Posterolateral	68	47– 98	0.08
	Anteromedial	344	156– 756	0.17
2	Posterolateral	49	25– 96	0.14
	Anteromedial	461	194–1092	0.17
3	Posterolateral	115	84– 157	0.05
	Anteromedial	417	139–1246	0.24
4	Posterolateral	20	12– 35	0.08
	Intermediate	62	42– 93	0.08
	Anteromedial	208	132– 326	0.09
5	Posterolateral	51	33– 80	0.10
	Intermediate	75	45– 125	0.11
	Anteromedial	231	88– 607	0.21
6	Posterolateral	34.5	23– 52	0.09
	Intermediate	54.5	41– 72	0.06
	Anteromedial	186.0	72– 484	0.21

TABLE VII

RELATIVE ABUNDANCE OF CELL TYPES IN THE HOG ANTERIOR PITUITARY

Gland No.	Tissue zone	Basophiles	Acidophiles	% Basophiles*
		Cells per field ± S.E.M.		
4	Posterolateral	32.3 ± 1.1	99.2 ± 8.4	24.5
	Intermediate	40.0 ± 0.5	71.7 ± 1.2	35.8
	Anteromedial	86.1 ± 1.7	19.2 ± 0.5	81.6
5	Posterolateral	44.5 ± 1.3	134.9 ± 1.9	24.8
	Intermediate	55.7 ± 0.7	63.5 ± 1.3	46.7
	Anteromedial	99.1 ± 1.5	33.5 ± 0.8	75.0
6	Posterolateral	43.9 ± 1.2	88.7 ± 1.8	33.2
	Intermediate	60.9 ± 1.1	72.4 ± 1.1	45.6
	Anteromedial	118.1 ± 1.8	28.7 ± 0.5	80.6

*Expressed as % of total stainable cells, i.e. basophiles + acidophiles.

Concentrations of ACTH in Rat, Beef, Hog, and Human Posterior Pituitaries

Table VIII shows that ACTH is present in the posterior lobes of whole rat and human pituitary glands and in two separated parts of beef and hog posterior pituitaries. The beef and hog glands were separated into the *crown*, which contains only the tissue remote from the anterior lobe, and the *base* adjacent to the anterior lobe (Fig. 1). There is no detectable difference between the ACTH contents of the crown and base. Histological examination revealed no anterior pituitary cells in the *crown*, although the *base* could conceivably contain some anterior lobe-like tissue admixed with the intermediate lobe tissue attached to the posterior lobe.

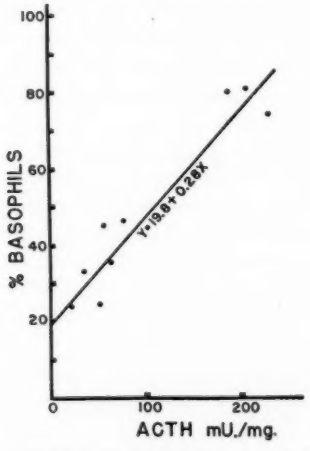

FIG. 3. Regression of the relative abundance of basophiles on the ACTH content in the hog anterior pituitary. The correlation coefficient is 0.96.

TABLE VIII

POSTERIOR LOBE ACTH

Gland	mU. ACTH per mg.	95% limits	Index of precision
Rat, whole	40.7	19–87	0.14
Rat, whole	23.3	10–53	0.16
Rat, whole	26.4	16–44	0.09
Rat, whole	36.2	29–44	0.04
Human, whole	24.6	15–40	0.10
Beef, crown	5.3	2–19	0.24
base	5.5	3–14	0.17
Hog, crown	43.8	26–74	0.11
base	16.3	9–31	0.14
Hog, crown	12.4	7–23	0.10
base	18.6	13–26	0.07
Hog, crown	12.6	9–18	0.07
base	15.0	8–29	0.15
Hog, crown	4.3	2–12	0.15
base	16.5	10–27	0.11
Hog, crown	20.8	12–37	0.12
Hog, crown	26.0	19–36	0.06
Hog, crown	14.6	12–18	0.04

Discussion

Much evidence suggests that the pituitary gland is under neural control (15). There are two possible communicating systems joining the brain to the pituitary: nervous and vascular. The posterior lobe of the pituitary gland consists largely of the endings of neurons originating in the nuclei of the hypothalamus, so that the posterior lobe is connected directly with other parts of the brain by nervous pathways. The ACTH in the posterior lobe is therefore accessible to a mechanism for direct nervous control of its release. In this connection, Mialhe-Voloss (28, 29) has suggested that neurotropic stresses, i.e., stimuli, such as sound, that influence the animal only via the nervous system, cause the discharge of the ACTH in the posterior lobe, without affecting the anterior lobe. There is no conclusive evidence for direct neural communication between the brain and the anterior lobe, so that neural control of the adenohypophysis is probably routed through vascular channels (15). These vascular connections include a portal system of capillaries that originate in the hypothalamus and terminate in the anterior lobe in a region adjacent to the pituitary stalk. This region, red in color because of its high vascularity, corresponds to the anteromedial zone in the hog gland (Fig. 1). It is reasonable to suggest that the part of the gland with the greatest concentration of ACTH would lie in the region vascularized by the portal capillaries. Tables V and VI show that the anteromedial zone, which is about one-fifth the mass of the anterior lobe, contains about one-half the total ACTH. In addition, this part of the anterior lobe would probably contain a relative abundance of the cell type responsible for the synthesis, storage, and release of ACTH. As shown in this paper, the anteromedial zone is rich in ACTH and basophiles, and the concentration of ACTH can be highly

significantly correlated with the relative proportion of basophiles in the various regions of the gland. These results point to the basophiles as the ACTH-containing cells in the glands of the species examined. In the rat, a species with a more diffuse distribution of basophiles, there is a more uniform distribution of ACTH (Fig. 2). Two subtypes of basophiles, the β- and δ-cells, have been described (14, 35). The available evidence is insufficient to ascribe with certainty the storage of ACTH to one of these types.

The presence of significant amounts of ACTH in the posterior lobe explains the "contamination" of posterior lobe hormone preparations by ACTH. The origin of the posterior lobe ACTH is puzzling because no typical basophilic cells can be detected in the posterior lobe. The ACTH may originate in either the neurosecretory cells of the hypothalamus or of the posterior lobe and may be stored in the posterior lobe for release by neurotropic stresses, just as the antidiuretic hormone is secreted after a stress (30, 31). Alternatively, the ACTH may originate in the anterior lobe and be transported to the posterior lobe by undefined vascular routes or by *post-mortem* diffusion. *Post-mortem* diffusion can be ruled out in the case of the rat, where the interval between decapitation and separation of the lobes is less than a minute while, in beef and hog pituitaries, there appears to be no concentration gradient between parts of the posterior lobe adjacent to and remote from the anterior lobe (Table VIII).

Morris *et al.* (32*a*) suggested that the ACTH in the human posterior lobe is formed by the cells in areas of "basophile cell invasion," which may represent the pars intermedia in man. The extraction and assay characteristics of the ACTH in the posterior lobe and in the anterior lobe are identical.

Acknowledgments

We are indebted to Mr. G. A. McDavid, Mr. R. S. Laws, and Mr. J Wyzykowski of Canada Packers Limited, Montreal, for providing hog and beef pituitary glands. The human tissue was obtained with the co-operation of Dr. Calvin Ezrin, University of Toronto.

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THE SECRETION OF IODIDE IN SALIVA[1]

A. S. V. Burgen and P. Seeman

Abstract

Iodide accumulation by the dog parotid gland continues during rest as well as during secretion. On stimulation of the gland this iodide appears as a brief transient iodide concentration that merges into a lower steady level. The amount of iodide accumulated depends on the duration of the rest period and reaches a limiting value with a half time of ~10 minutes. Both transient and steady state saliva/plasma concentration ratios are readily reduced by increased plasma I' and by ClO_4'. The residual iodide secretion after depression by ClO_4' behaves like iodide secretion by the submaxillary gland. This gland has no iodide concentrating system. Iodide clearance values are high and of the order of the estimated total blood flow. Evidence is presented that iodide is concentrated by the duct system and that the ducts have a very high blood flow.

The excretion of iodide in saliva after injection in the circulation seems first to have been studied by Bernard (2). He noted that iodide could still be detected in the saliva after it had apparently disappeared from the blood. He thus showed that a greater concentration of iodide could be present in the saliva than in the plasma. Quantitative studies by Lipschitz (7) established the degree of concentration occurring in dog parotid saliva and established that the saliva/plasma (S/P) ratio could be depressed by an increase in plasma iodide level.

More recently Rowlands, Edwards, and Honour (11) have studied iodide secretion by the human parotid gland and found that the S/P ratio for tracer iodide was depressed by both thiocyanate and perchlorate ions that are themselves concentrated in the saliva (5). Fletcher, Honour, and Rowlands (6) have also made a very interesting study of iodide accumulation by salivary gland slices which agrees well with deductions from the *in vivo* studies.

The similar properties of the iodide-concentrating mechanisms of the salivary glands and the thyroid have aroused a great deal of interest and several authors have examined both saliva and gland homogenates for organically bound iodide without positive result (6, 8, 11). It appears that the salivary glands can accumulate inorganic iodide but, unlike the thyroid, are unable to incorporate it into organic molecules.

Recently Logothetopoulos and Myant (8) have shown by radioautography of freeze-dried mouse and hamster salivary glands that the concentration of iodide and thiocyanate occurs exclusively in the secretory ducts and not in the acini.

The present work is concerned with a dynamic study of the iodide-concentrating mechanism in the dog's parotid gland and offers support for the view that the iodide concentration occurs in the duct system. An important consequence of this is that the very high rate of iodide clearance by the gland entails a correspondingly high rate of blood flow through the capillaries enveloping the ducts.

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Contribution from the Department of Physiology, McGill University, Montreal, Que.

Experimental

Adult mongrel dogs were anesthetized with chloralose–urethane mixture and prepared for saliva collection (3, 4). Thiouracil (100 mg./kg.) was given intravenously to block organic incorporation of iodide. In long experiments booster doses of thiouracil (50 mg./kg.) were given every 4 hours. Carrier-free I^{131} was given intravenously in all experiments and, where higher iodide levels were desired, a rapid infusion of 5% NaI was given followed by a slower sustaining infusion. At least 30 minutes was allowed for equilibration after a shift to a new level. Samples of plasma and saliva were plated in amounts of 0.1–0.2 ml. on 27 mm. aluminum dishes, dried, and counted with a thin end-window GM tube (Tracerlab TGC2). Appropriate corrections were made for self absorption and radioactive decay. Total iodide in plasma and saliva was measured by the method of Alpert (1) and chloride by the method of Van Slyke and Hiller (13).

Results

In view of previous experience with potassium secretion in saliva (4) in which a time dependence in concentration existed when the gland was suddenly thrown into activity after a period of rest, a similar time dependence was anticipated with iodide. Accordingly small successive samples of saliva were collected at each rate of secretion. In Fig. 1, the dog had received tracer amounts of I^{131} 2 hours previously and secretion was produced by supra-maximal stimulation of the auriculotemporal nerve at 7 c.p.s. In the experiment shown in the upper curve a 24 minute period had elapsed since the previous period of stimulation. The iodide S/P in the saliva rapidly rose to a peak of 35 at 0.32 minutes after stimulation began and then declined to steady level of 5.6. In the experiment shown in the lower curve the rest period since previous stimulation was only 5 minutes. The transient level of S/P now reached only 12.2 but the steady level was 6.0, approximately the same as before. Note that the form and duration of the transient were very similar in the two runs; only the magnitude was changed. Other experiments have confirmed that an initial peak of iodide concentration whose magnitude was dependent on the rest period was invariably found in parotid secretion.

For a quantitative study of the transient it is more satisfactory to convert it into an *amount* instead of a concentration by integrating the total iodide output and subtracting from it the amount that would have been secreted if the entire iodide output had been secreted at the steady state level, i.e.,

$$\sum_0^t \frac{VS}{P} - \left(\frac{S}{P}\right)_s \sum_0^t V$$

where V = rate of saliva secretion, $(S/P)_s$ = steady state ratio. The value so obtained is the amount of iodide (Q) relative to the plasma level accumulated by the gland during the rest period.

This value, Q, is independent of the rate of saliva secretion (i.e. of the rate of nerve stimulation) and dependent only on the rest period.

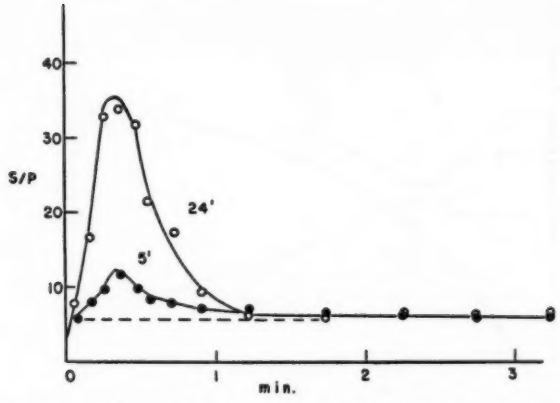

FIG. 1. Parotid secretion. Dog. 15 kg. Stimulation at 7 c.p.s. O—O Twenty-four minute rest since previous period of stimulation. ●—● Five minute rest since previous stimulation.
Ordinate: saliva/plasma concentration ratio.
Abscissa: time in minutes.

In Fig. 2 the relationship between Q and rest period may be seen. At first Q increases rapidly with time but then levels off and tends towards a limiting value. The curve was drawn for an equation of the form

$$Q = Q_\infty(1-\exp(-\alpha t)) \qquad (1)$$

with $Q_\infty = 6.0$ and $\alpha = 0.066$ minute^{-1} and gives a good fit. The transient thus reaches a steady value with a half time of 10.5 minutes.

The duration of the iodide transient in Fig. 1 was only 0.22 minute (measured at 90% of peak height), and since the rate of saliva secretion in this experiment was 0.265 ml./g. minute the transient may be regarded as occupying a fluid volume of 0.058 ml./g. gland.

Measurement of the volume occupied by the ducts in formalin-fixed, paraffin embedded sections by microscopic examination gave values of 5–10% of the gland volume. The similarity of this volume to that occupied by the iodide transient is consistent with the iodide excreted in the transient being present in the ducts and then being ejected in the column of fluid. In the experiment shown in Fig. 1 the delay in reaching the peak was 0.21 minute. This is slightly longer than usual and can be partly accounted for by the latency before secretion starts to flow (~0.05 minute) and the dead space of the cannula and main secretory duct (~0.08 minute). The remaining delay may be due to the lack of concentrating power of the collecting ducts (cf. 8).

The second curve in Fig. 2 shows the variation with time of plasma clearance of iodide calculated by dividing the duration of the rest period by t. At short intervals the plasma clearance is reasonably constant but falls off with increase in the rest period, presumably because of back diffusion of iodide from the duct lumen into the blood as the concentration in the duct lumen increases.

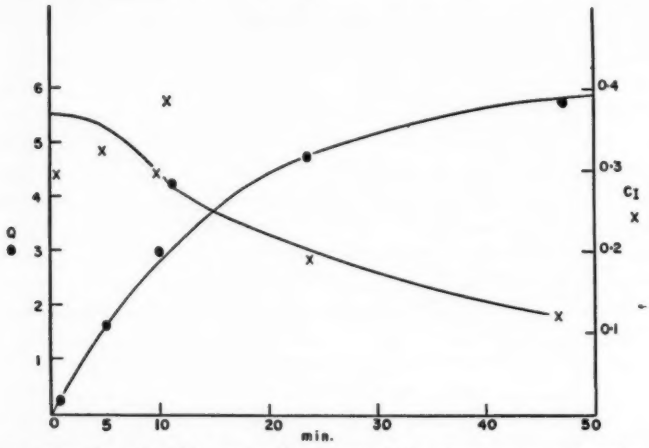

FIG. 2. Quantity of iodide accumulated and iodide clearance as a function of rest period. ●—● Accumulated iodide. Solid line $Q = 6.0\,(1 - \exp(-0.066t))$. ×—× Iodide plasma clearance. Solid line $C_I = 0.396.\exp(-0.066t)$.
Ordinate left: accumulated iodide = ml. plasma/g. gland.
Ordinate right: plasma clearance in ml./g. minute.
Abscissa: rest period in minutes.

The initial clearance may also be obtained by differentiating equation (1) giving

$$\frac{dQ}{dt} = Q_\infty \,.\, \alpha \,.\, \exp(-\alpha t)\,. \qquad (2)$$

At $t = 0$ this equals $Q_\infty \alpha$, which is the initial clearance rate. Thus with $Q_\infty = 6.0$ and $\alpha = 0.066$ the initial clearance is 0.396 ml./g. minute. In various experiments this calculation led to a clearance of 0.25–0.45 ml. plasma/g. minute. Conversion of plasma clearances to whole blood clearance offers some difficulty because of the rapid equilibration of iodide between plasma and red cells. Tosteson (12) has measured this rate of equilibration, and extrapolation of his figures to 37° C. gives a half time of the order of 4 seconds. This would probably permit partial equilibration in the capillaries of the gland. The distribution of iodide between plasma and cells is such that red cell iodide is about 66% of plasma iodide (10) so that whole blood clearance would be between 1.2–2 times as great as the plasma clearance. We may estimate the resting whole blood clearance as 0.30–0.60 ml./g. minute.

Effect of Raised Plasma Iodide Level on Transient and Steady State S/P Ratios

When the plasma iodide level was raised by infusion of sodium iodide to give a plasma iodide level in excess of about 0.5 meq./liter, depression of both the transient and steady state S/P ratios occurred. Figs. 3 and 4 show such experiments. In Fig. 3 with tracer iodide levels the transient S/P reached 50 after a rest of 60 minutes and the steady state S/P was 4.0; when the plasma iodide was raised to 0.82 meq./liter the transient S/P fell to 12 and the steady state to 2.9. When the plasma iodide was raised further to 13.8 meq./liter the transient S/P was only 3.1 and the steady state S/P was now only 0.69.

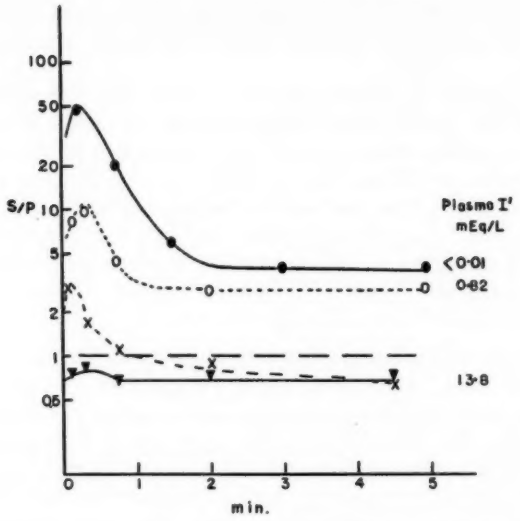

FIG. 3. Iodide transient and steady state. Effect of increased plasma iodide. ●—● Tracer iodide. ○--○ 0.82 meq./l. I'. ×--× 13.8 meq./l. I'. ▼—▼ 13.8 meq./l. I' + 2 meq./l. ClO_4'. Stimulation 7 c.p.s.; rest period between stimulations, 60 minutes.
Ordinate: iodide S/P.
Abscissa: time in minutes.

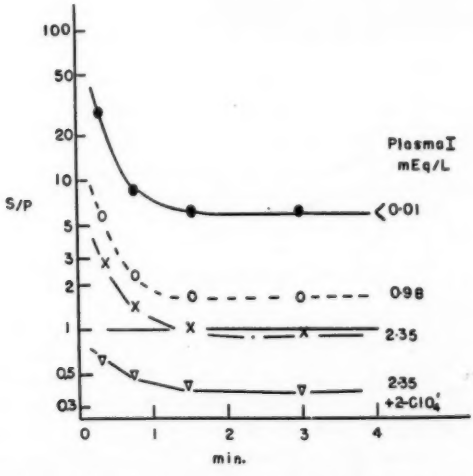

FIG. 4. Iodide transient and steady state. Effect of increased plasma iodide. ●—● Tracer iodide. ○--○ 0.98 meq./l. I'. ×—× 2.35 meq./l. I'. ▽—▽ 2.35 meq./l. I' + 2 meq./l. ClO_4'. Stimulation 10 c.p.s.; rest period between stimulations, 40 minutes.
Ordinate: iodide S/P.
Abscissa: time in minutes.

Injection of 100 mg./kg. of $NaClO_4$, giving a plasma perchlorate of $\sim$ 2 meq./liter, did not reduce the steady state level further but practically abolished the transient.

In Fig. 4 a similar situation is found. With tracer amounts the steady state S/P was 5.8, with a plasma iodide of 0.98 meq./liter it was reduced to 1.6 and with a plasma iodide of 2.35 meq./liter this was further reduced to 0.90. On injection of 100 mg./kg. of $NaClO_4$ it settled down to S/P = 0.41. Higher doses of perchlorate produced no further depression.

It is evident that the *amount* of iodide that can be transported at rest and in the steady state is limited and, because of this limitation, when the plasma iodide is raised sufficiently, the S/P ratio is depressed. However, the limiting value reached is not 1 but significantly less than 1.

Relationship between Steady State Secretion of Iodide and Rate of Saliva Secretion

The S/P ratio for iodide is relatively independent of the rate of saliva secretion. There is, however, some increase in ratio at very low flow rates and occasionally, as in the experiment shown in Fig. 5, a slight increase at the highest flow rates. With increase of the plasma iodide concentration the whole curve is shifted downwards and sometimes, but not invariably, the depression is greater at the high flow rates. With very high plasma iodide concentrations the S/P over the whole range is depressed below 1.

Iodide Secretion by the Submaxillary Gland

The submaxillary gland of neither the dog nor the cat is able to concentrate iodide. In other species (mouse and hamster (8)) the submaxillary gland is more active than the parotid. In the rat (8), however, none of the salivary glands appear to be active in transporting iodide.

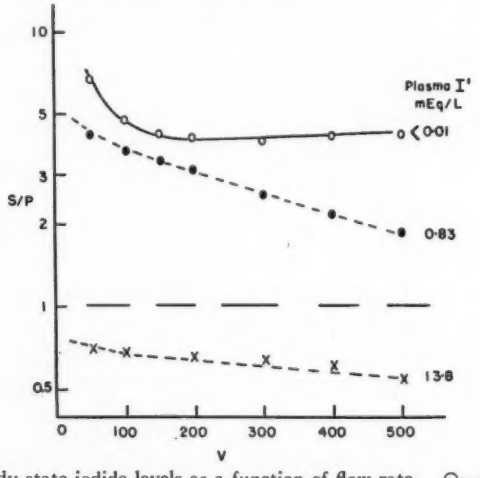

FIG. 5. Steady state iodide levels as a function of flow rate. O—O Tracer iodide. ●—● 0.83 meq./l. I'. ×--× 13.8 meq./l. I'.
Ordinate: iodide S/P.
Abscissa: rate of secretion mg./g. minute.

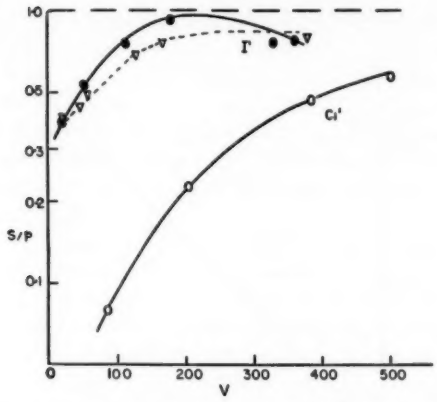

FIG. 6. Iodide concentration in submaxillary gland. Steady state values. ●—● Parotid iodide, plasma iodide 2.3 meq./l.; ClO_4' ~ 2 meq./l. ○—○ Parotid chloride. ▽--▽ Submaxillary iodide in same animal.
Ordinate: S/P ratio.
Abscissa: rate of secretion in mg./g. minute.

The submaxillary saliva of both dog and cat do contain iodide but at a level less than that in the blood. This S/P ratio was unaffected by raising the plasma iodide and was not usually affected by large doses of perchlorate. In some animals the submaxillary iodide level was slightly *increased* by perchlorate. After the administration of perchlorate the iodide level in both submaxillary and parotid saliva became similar as can be seen in Fig. 6. The iodide levels in the saliva from both glands were considerably higher than the chloride level and did not show a similar relationship to flow.

Iodide Clearance by the Parotid during Secretion

Iodide clearances may be calculated at any time during steady state secretion. In one experiment (Fig. 7) these ranged from 0.25 ml. plasma/g. minute at rest to 2.2 ml./g. minute when the rate of secretion was 0.5 ml./g. minute. The corresponding blood clearance at this rate of secretion would be 2.7–4.4 ml./g. minute.

Discussion

The concentration of iodide by the dog's parotid gland occurs during rest as well as during activity; indeed the present experiments yield no evidence of a specific effect of activation of the gland on iodide transport. It seems quite probable that the increased iodide clearance that occurs during activity is merely an indication of the increased blood flow and hence availability of iodide ion. The values for iodide clearance in the parotid gland are of the same order as the total blood flow to the dog submaxillary gland at equivalent secretion rates. We found (3) that the maximum blood flow in the dog submaxillary gland was about 2.6 ml./g. minute and that, at high rates of flow, a blood flow/saliva secretion rate of 4–7 prevailed. There are indications

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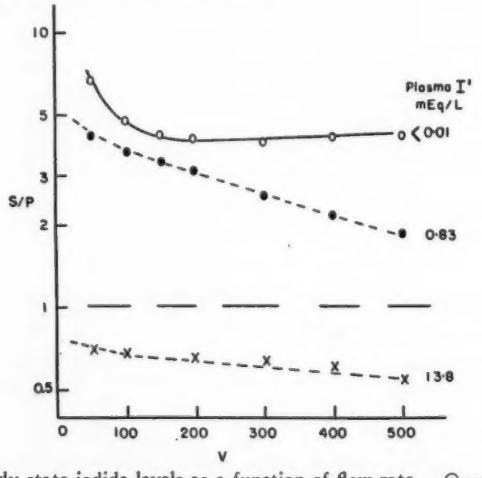

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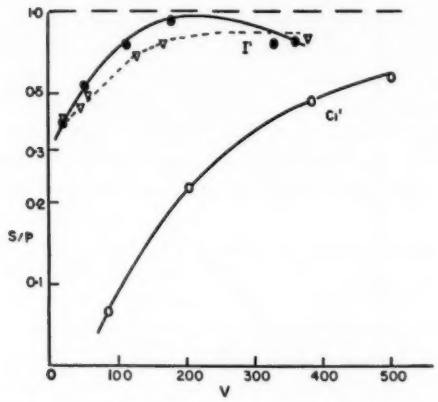
Ordinate: iodide S/P.
Abscissa: rate of secretion mg./g. minute.



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Ordinate: S/P ratio.
Abscissa: rate of secretion in mg./g. minute.

The submaxillary saliva of both dog and cat do contain iodide but at a level less than that in the blood. This S/P ratio was unaffected by raising the plasma iodide and was not usually affected by large doses of perchlorate. In some animals the submaxillary iodide level was slightly *increased* by perchlorate. After the administration of perchlorate the iodide level in both submaxillary and parotid saliva became similar as can be seen in Fig. 6. The iodide levels in the saliva from both glands were considerably higher than the chloride level and did not show a similar relationship to flow.

Iodide Clearance by the Parotid during Secretion

Iodide clearances may be calculated at any time during steady state secretion. In one experiment (Fig. 7) these ranged from 0.25 ml. plasma/g. minute at rest to 2.2 ml./g. minute when the rate of secretion was 0.5 ml./g. minute. The corresponding blood clearance at this rate of secretion would be 2.7–4.4 ml./g. minute.

Discussion

The concentration of iodide by the dog's parotid gland occurs during rest as well as during activity; indeed the present experiments yield no evidence of a specific effect of activation of the gland on iodide transport. It seems quite probable that the increased iodide clearance that occurs during activity is merely an indication of the increased blood flow and hence availability of iodide ion. The values for iodide clearance in the parotid gland are of the same order as the total blood flow to the dog submaxillary gland at equivalent secretion rates. We found (3) that the maximum blood flow in the dog submaxillary gland was about 2.6 ml./g. minute and that, at high rates of flow, a blood flow/saliva secretion rate of 4–7 prevailed. There are indications

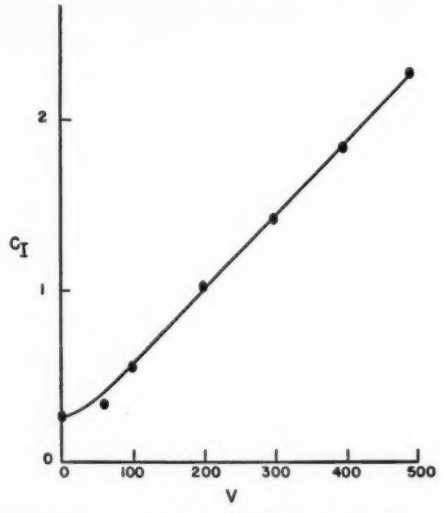

FIG. 7. Iodide clearance as a function of rate of saliva secretion. Tracer iodide.
Ordinate: plasma iodide clearance, ml./g. minute.
Abscissa: rate of secretion, mg./g. minute.

in terms of the greater stability of the gland potassium level and greater rate of replenishment after depletion in the parotid that this gland has a rather higher blood flow/saliva ratio than in the submaxillary gland (Burgen and Seeman, to be published), but nevertheless the iodide clearance probably accounts for a large fraction of the total gland flow. Unfortunately it has not been found possible to perform satisfactory isolation of the parotid circulation in either the dog or cat in order to obtain a direct measurement of the blood flow or iodide extraction.

The time course and volume of distribution of the iodide transient suggests that the iodide is secreted into a duct segment that possibly does not include the collecting ducts. This is in close agreement with the radioautographic studies of Logothetopoulos and Myant (8) in which the iodide was concentrated solely in the columnar duct segment. The results of these authors were especially striking in the mouse and hamster parotid glands in which the *whole gland* radioactivity was lower than that in the plasma and yet, over the columnar duct segments, an intense autographic reaction was obtained. In these experiments it was not possible to be certain that all the radioactivity was in the duct lumen and it seemed possible that a two stage blood → cells → saliva concentrating system was present. A reservoir in the duct cells might explain the not infrequent trailing of the iodide transient, i.e. the main transient might represent iodide lying free in the duct lumen and, when this is swept out, the iodide within the duct cells is transferred to the lumen and added to the trailing part of the transient. The evidence just cited agrees in attributing the transfer of iodide exclusively to the ducts.

A very important consequence of this is that the *minimum blood flow in contact with the ducts* is that given by the iodide clearance. As the ducts occupy not more than 10% of the gland volume the minimum duct blood flow must reach the very large figure of 20–30 ml./g. duct/minute. In the absence of reliable figures for total gland flow it is not possible to compare the acinar blood flow with the duct flow, but other evidence (Burgen and Seeman, in preparation) suggests that the blood perfusing the ducts also perfuses the acini through a portal system.

The iodide transport system in the salivary glands seems typical of many active ion transport systems in that it is readily saturated by an increase in the concentration of ion in the source fluid, depressed by competitive ions (i.e. ClO_4'), and can support only a limited steady state gradient (in this case 50–100 : 1). On the other hand the secretion of iodide in the dog and cat submaxillary glands and the residual iodide secretion by the parotid after self saturation and depression by perchlorate bear the marks of a passive mechanism.

In view of the similarity of the hydrated ion radii of chloride and iodide it is perhaps surprising that the ions do not behave more alike, but it should be noted that differences of treatment of the two ions are found in the red cell (9, 11) and kidney (8) under conditions suggestive of passive anion movement.

Acknowledgments

We are grateful to the National Research Council for generous support and to Mrs. N. Hildebrand for unfailing technical assistance.

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THE MEASUREMENT OF THE RATE OF BLOOD FLOW IN THE CALF AND PAW OF DOGS BY THE VENOUS OCCLUSION PLETHYSMOGRAPH TECHNIQUE WITH A NOTE ON THE EFFECTS OF INTRAVENOUS ADRENALINE AND NORADRENALINE[1]

J. D. Hatcher and D. B. Jennings

Abstract

Anatomical and physiological evidence is presented which indicates that venous occlusion plethysmographic measurements of blood flow in the calf and in the paw of dogs give an index of muscle and skin blood flow respectively.

The venous occlusion plethysmograph technique has been used extensively to measure the rate of blood flow in the extremities of man and in particular in the forearm, calf, hand, and foot. On the basis of dissections of limb segments and a vast amount of physiological investigation, it is generally conceded that measurements of blood flow by this method in the forearm or calf and in the hand or foot give a measure of muscle and skin blood flow respectively.

This paper is concerned with anatomical and physiological data obtained in dogs which indicate that the plethysmographic method of blood flow measurement in the calf and in the paw give a measure of muscle and skin blood flow respectively.

Methods

Nine 4-in. calf segments, measured peripherally from the knee articulation, were removed from dead dogs. After the hair was removed with clippers the segments were dissected into their component tissues: (1) skin with as much subcutaneous fat removed as possible without disturbing the dermis, (2) muscle, (3) tendon and fascia, (4) bone, and (5) fat, to which was added blood vessels, nerves, and lymph glands. Evaporative water loss was minimized by covering the segments and pieces of tissue with cloths dampened with saline. The per cent by weight and by volume of the whole segment and of each component tissue was determined.

Six 4-in. terminal or paw segments were studied similarly. Nails were included with the bone, and the footpads, with as much fat removed as possible, were included with the skin.

These segments of calf and paw correspond to the areas enclosed by the plethysmographs.

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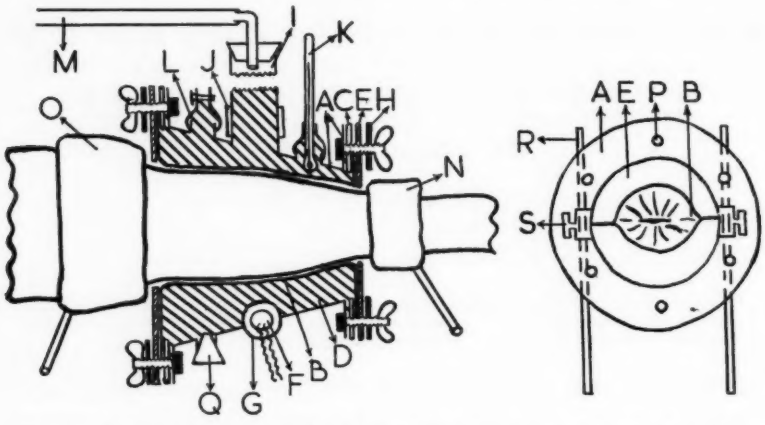

FIG. 1. The calf plethysmograph (A) was made from $\frac{1}{8}$ in. brass. The loose rubber sleeve (B) was constructed from a meteorological balloon. Each end of the sleeve was everted and glued to a gasket of inner tire tube rubber (C). The hydrostatic pressure of the water (D) between the rubber sleeve and the plethysmograph forced the loose sleeve in folds against the powdered skin and against the inner surface of linoleum plates (E) at either end. The linoleum plates were shaped to fit closely but not tightly about the calf thus preventing outward movement of the rubber sleeve and ensuring that volume changes were directed to the float recorder. The rubber gasket (C) and linoleum plates (E) were held tightly against the flange of the plethysmograph (A) by a metal ring (H) and wing nuts (P). The level of the water in the glass chimney (I) which was connected by rubber tubing (J) to the plethysmograph was kept 1–2 inches above the plethysmograph. Bubbles of trapped air were allowed to escape through opening (L). Running transversely across the bottom of the plethysmograph was a hollow tube (G) containing a soldering iron element (F) connected to a variable resistance. Water temperature recorded by thermometer (K) was kept constant by this element. Water could be emptied through opening (Q). An arterial occluding pressure cuff (N) was placed below the plethysmograph and a venous occluding pressure cuff (O) above the plethysmograph. The cuffs were wrapped with bandage and were inflated from a foot pump – reservoir system. The paw plethysmograph is similar to the calf plethysmograph except that the distal end of the metal cylinder and loose rubber sleeve are closed.

Details of construction of the plethysmographs are shown in Fig. 1. Muscle temperature under the calf plethysmograph was kept at 35.0° C. with a water temperature of 34 ± 0.5° C. The paw plethysmograph was placed on the forelimb. The water temperature in the paw plethysmograph was kept at 32 ± 0.5° C. The theoretical basis of the method has been described by others (1).

Blood flow measurements were carried out on dogs over 15 kg. in weight anaesthetized with sodium pentobarbital. An initial dose of 30 mg./kg. was given intravenously followed by 60–120 mg. intramuscularly every 1–2 hours as necessary. Measurements were begun 2–2½ hours after the initial dose of anaesthetic. Room temperature was 24.0 ± 1.5° C. The arterial cuff below the calf plethysmograph was inflated at 250 mm. mercury pressure 15 seconds before inflation of the venous cuffs above the plethysmographs. The venous cuffs were inflated at 50–70 mm. mercury pressure for 5 to 10 seconds, and the limb volume changes produced were recorded with float recorders writing on moving kymograph paper. The venous cuffs were then deflated for about

5 seconds during which time the levers of the float recorders returned to base line; the cuffs were then inflated again. This cycle of venous cuff inflation and deflation was repeated for 2 minutes out of every 5 minutes. At the end of this 2 minute period both venous and arterial cuffs were deflated. The rate of blood flow in cc./100 cc. of tissue/minute was calculated from the slope of the volume change by the method of Barcroft and Edholm (3). The individual blood flow records in each 2 minute period were averaged to give the mean blood flow for that period. The calf and paw blood flow was measured in this manner in eight dogs over a 45–60 minute period. The effect of intravenous infusions of adrenaline and of noradrenaline on the calf and paw blood flow will be briefly described in order to indicate the sensitivity of the method. The effect of these agents on peripheral blood flow will be discussed in more detail in a subsequent publication (10).

Results and Discussion

The results of the dissection studies are shown in Table IA. The dissection data obtained by Cooper and colleagues (5) and Abramson and Ferris (2) on human forearm and hand segments are given in Table IB. A close agreement is noted between the ratio of muscle to skin in the calf of the dog (5 : 1) and the forearm of man (6 : 1). In the paw of the dog there is four times more

TABLE I

(A) PER CENT BY WEIGHT AND VOLUME OF COMPONENT TISSUES IN 4-IN. SEGMENTS OF THE CALF AND PAW OF THE DOG

	Skin		Muscle		Tendon		Fat		Bone	
	% by wt.	% by vol.	% by wt.	% by vol.	% by wt.	% by vol.	% by wt.	% by vol.	% by wt.	% by vol.
Mean of 9 calf segments	10.6	10.7	55.5	55.8	2.5	2.6	10.2	10.4	21.2	20.5
S.D.	±3.76	±3.56	±5.74	±5.82	±2.34	±2.54	±4.78	±5.12	±4.15	±4.81
S.E.M.	±1.25	±1.19	±1.91	±1.94	±0.78	±0.85	±1.59	±1.71	±1.38	±1.60
Mean of 6 paw segments*	30.9	32.9	8.0	7.9	6.4	6.4	11.9	12.0	42.8	40.8
S.D.	±5.12	±5.51	±2.01	±1.93	±1.51	±1.62	±5.04	±4.80	±3.84	±4.39
S.E.M.	±2.09	±2.25	±0.82	±0.79	±0.62	±0.66	±2.06	±1.96	±1.57	±1.79

(B) MEAN PER CENT BY VOLUME OF COMPONENT TISSUES IN FOREARM AND HAND OF MAN†

Segment	Skin	Muscle	Fat, tendon, and bone
Forearm	9.9	61.1	29.0
Hand	30.2	15.5	54.3

*The six paw segments included three from the foreleg and three from the hindleg. No significant difference was noted in the volume of component tissues from the two sites.

†Forearm data are an average of those presented by Cooper *et al.* (5) and Abramson and Ferris (2). The hand dissection data are from Abramson and Ferris (2).

skin than muscle, whereas in the hand of man there is only twice as much skin as muscle. This difference between the two species is accounted for by a greater volume of muscle in the human hand.

Since the plethysmographic method does not measure bone blood flow (1), and since fat and tendon are relatively avascular (1), it may be concluded on anatomical grounds that the calf blood flow will represent largely muscle blood flow and the paw blood flow essentially skin blood flow.

The average calf blood flow in eight anaesthetized dogs was 5.5 cc./100 cc. calf/minute and the average paw blood flow was 13.4 cc./100 cc. paw/minute (Table II). From a scattergram presented by Freeman and Zeller (7) it can be estimated that with a water temperature in the plethysmograph of 32.0° C. the paw blood flow in the sympathectomized limb of trained unanaesthetized dogs was approximately 11.0 cc./100 cc. paw/minute. We have been unable to find other data for the blood flow in the calf of the dog or for muscle blood flow expressed in terms of muscle mass. The normal rate of blood flow in the extremities of man is determined at a comfortable environmental temperature, for the clothing worn, and with the water in the plethysmographs at 'neutral' temperatures which vary with the part under study (1, 3). Since pentobarbital anaesthesia affects temperature regulation (8, 9) it is difficult to know if these conditions have been met in the present experiments even though the rectal temperature and tissue temperatures under the plethysmographs were maintained fairly constant by the use of table heat, blankets, and the water in the plethysmographs. The average rate of blood flow in the calf and paw of dogs is greater than the average stated for comparable areas in man (1, 3, 6). This difference must be interpreted in the light of the effects of the anaesthetic

TABLE II

CALF AND PAW BLOOD FLOW IN EIGHT DOGS ANAESTHETIZED WITH SODIUM PENTOBARBITAL

	Calf blood flow, cc./100 cc. calf/minute			Paw blood flow, cc./100 cc. paw/minute		
Dog No.	Mean	S.D.	S.E.M.	Mean	S.D.	S.E.M.
1	3.10	±2.23	±0.48	8.80	±3.12	±0.70
2	7.63	±1.28	±0.43	16.28	±2.82	±1.05
3	5.58	±1.05	±0.40	14.63	±1.21	±0.46
4	2.75	±0.49	±0.17	12.11	±3.03	±1.07
5	6.28	±1.79	±0.63	13.41	±2.22	±0.79
6	4.12	±0.66	±0.18	9.15	±0.80	±0.22
7	10.17	±1.65	±0.46	17.25	±1.95	±0.54
8	4.55	±0.48	±0.13	15.53	±1.80	±0.50
Mean	5.5			13.4		
S.D.	±2.48			±3.14		
S.E.M.	±0.88			±1.11		

NOTE: The mean calf and paw blood flow with standard deviation and standard error are given for each dog. These values are based on recordings made over a 45–60 minute period. The mean calf and paw blood flow with the standard deviation and standard errors for the entire group of eight dogs are shown at the bottom of the table.

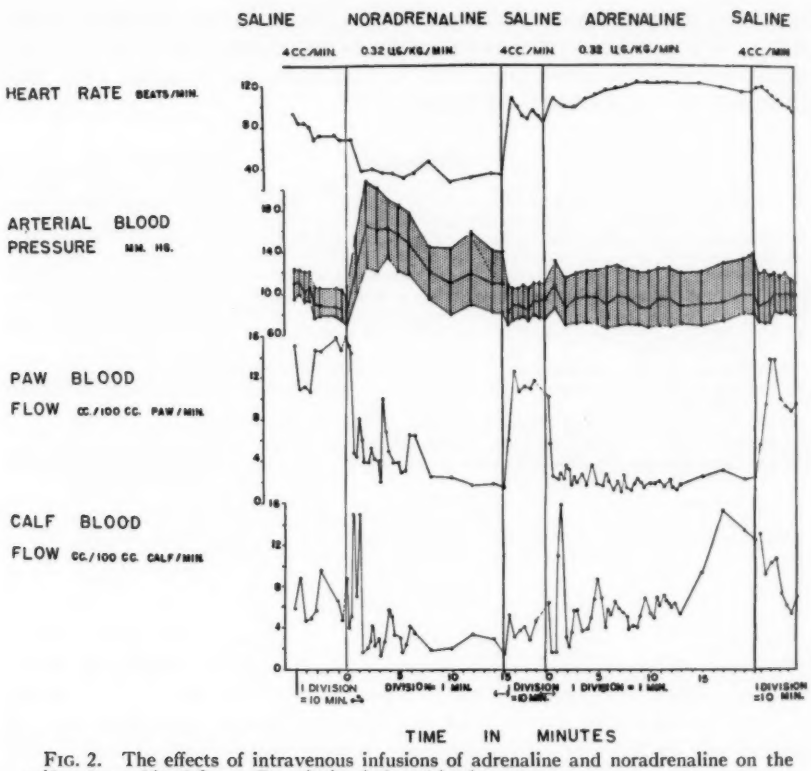

FIG. 2. The effects of intravenous infusions of adrenaline and noradrenaline on the calf and paw blood flow. Description is found in the text.

not only on temperature regulation, but also on the peripheral vasculature (8, 9, 11). The forearm or calf blood flow in man is influenced by, and reflects changes in, the metabolic activity of skeletal muscle (1). The hand or foot is composed largely of skin which has a much lower metabolic activity than muscle, and the blood flow in these areas, rather than reflecting the metabolic activity of the most abundant tissue, is mainly concerned with heat dissipation and conservation (1). The greater rate of blood flow in the paw as compared with the calf of the dog may be related to similar factors.

In Fig. 2 are shown the effects on calf and paw blood flow of intravenous infusions of adrenaline and of noradrenaline at a rate of 0.32 μg./kg./minute for 15–20 minutes. The results are remarkably similar to those described for the forearm and hand of man (4). Both noradrenaline and adrenaline caused a reduction in paw blood flow. Noradrenaline produced an initial transient increase in calf blood flow followed by a prolonged decrease lasting for the remainder of the infusion. Adrenaline produced a similar initial transient rise in calf blood flow followed by a fall to control levels, after which the blood flow gradually increased. A more complete description of the effects of these

agents on the peripheral circulation will be presented in a subsequent paper (10). This experiment, however, clearly demonstrates that the calf plethysmograph measures blood flow in a vascular bed different from that measured by the paw plethysmograph.

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THE EFFECT OF GROWTH HORMONE ON THE UTILIZATION OF 1-C^{14} OCTANOIC ACID BY RAT LIVER SLICES[1]

W. F. Perry and H. G. Friesen

Abstract

Young (1–1½ months), adult (4–5 months), and old (2(+) years) rats were injected with growth hormone intraperitoneally in doses of 4 mg./100 g. at various intervals of time before removal of the liver. Slices of liver were incubated with radioactive octanoic acid and the production of CO_2 and acetoacetic acid measured.

In adult rats fed ad libitum, growth hormone injected 4 hours before the rats were killed had no consistent effect on acetoacetic acid or carbon dioxide production by the liver slices. In adult rats fasted 24 hours before they were killed, growth hormone was likewise found to have no effect on ketogenesis and CO_2 production irrespective of whether it was injected 4, 12, or 24 hours before the rats were killed. Young rats that were fasted 24 hours and to which growth hormone was administered at the 20th hour of fasting showed a slight ketogenesis but the values for the specific activity of the acetoacetic acid suggested the increased ketogenesis was not derived from the labelled fatty acid. No effect on CO_2 production was noted. In old rats that were fasted 24 hours and to which growth hormone was given at the 20th hour of fasting, a slight decrease in acetoacetic acid formation by the liver slices was observed which appeared to be due to an over-all reduction in fatty acid utilization. There was again no alteration in CO_2 production. Treatment of adult rats for 5 days with growth hormone, followed by incubation of the liver slices with octanoate, was found to influence neither ketogenesis nor CO_2 production.

Introduction

Among the endocrine glands, the anterior pituitary has long been implicated in the metabolism of fat. Hypophysectomy was shown by Li, Simpson, and Evans (11) to cause an accumulation of fat in the depots as well as a decrease in body nitrogen. Several investigators have shown that administration of anterior pituitary extracts depletes the fat depots and increases the amount of liver fat. In addition, anterior pituitary extracts have been shown to enhance ketogenesis by surviving liver slices (5, 13, 15). When purified growth hormone became available it was considered that it might be the pituitary factor concerned with fat metabolism. Growth hormone injections were observed to markedly increase liver lipids with parallel depletion of depot fats (4, 8, 11, 17), and various investigators have reported growth hormone to be ketonemic (2, 3, 12).

The source of the ketonemia is not clear. Bennett *et al.* (2), using the eviscerated animal, obtained data which indicated that the ketonemic action of growth hormone could not be a result of decreased peripheral utilization of ketone bodies. Enhancement of ketone body formation by liver preparations in growth hormone treated animals has been reported by some workers (12, 16) but not by others (2, 3).

The present experiments were undertaken in an attempt to further clarify the ketogenic action of growth hormone by studying its effect on the utilization of radioactive fatty acid by surviving rat liver slices.

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Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Manitoba.

Methods

The experiments were done on Wistar strain male albino rats. As preliminary work had suggested that there might be some difference in action of growth hormone between young and older animals, three age groups of rats were investigated—young (1–1½ months of age), adult (4–5 months old), and old (2(+) years old). Both fasting animals and animals fed ad libitum were used with appropriate controls. Crystalline growth hormone (Connaught Laboratories) was dissolved (NaOH solution, pH 8.0) immediately prior to injection. It was injected intraperitoneally in doses of 4 mg./100 g. at various time intervals before the animal was killed.

The animals were killed by decapitation and 1–1½ g. of liver slices were quickly prepared, using a Stadie-Riggs hand microtome, and suspended in 10 ml. of a modified Krebs–Ringer phosphate buffer, pH 7.4, having a K^+ concentration of 70 meq./liter. To the medium was added 2 μc. 1-C^{14}-labelled sodium octanoate, the total concentration of octanoic acid being 0.42 mg./ml. The flask was incubated, with shaking, for 3½ hours at 37° C., at the end of which time 1 ml. 5 *N* HCl was added. The carbon dioxide produced was trapped in 30% KOH (made up to a suitable volume), the carbonate was precipitated with barium chloride, and infinitely thick plates of barium carbonate were counted. The medium was deproteinized and then analyzed for acetone by the method of Greenberg and Lester (10), following the aniline-catalyzed decarboxylation of acetoacetic acid by the method of Edson (6). The CO_2 resulting from the decarboxylation was trapped in alkali and precipitated as $BaCO_3$ and its radioactivity determined, thus giving a measure of the incorporation of the octanoate carbon into acetoacetic acid.

In all experiments, preparations from a pair of animals, control and treated, were set up simultaneously and statistical analysis done on the differences between pairs.

Results

In Table I are shown data on acetoacetic acid and CO_2 production by liver slices from adult rats. It will be seen that, in animals fed ad libitum, growth hormone given 4 hours before the animals were killed did not cause any increase in acetoacetic acid production by the liver slices; nor was there any evidence of an increased incorporation of octanoic acid carbon into acetoacetic acid or carbon dioxide. When similar animals were subjected to a 24 hour fast (growth hormone being given at the 20th hour of the fast), there was no significant difference in any of the measurements between the control and the growth hormone treated animals, although the expected increased ketogenesis of fasting occurred.

Greenbaum and McLean (9) have reported growth hormone to be maximally active in stimulating a liver fatty acid oxidase system 12–24 hours after injection of growth hormone. We investigated the effect of growth hormone on animals fasted 24 hours when the hormone was given 12 and 24 hours before the animals were killed, and given also following 5 days' treatment with

TABLE I

THE EFFECT OF GROWTH HORMONE ON ACETOACETIC ACID AND CO_2 PRODUCTION BY ADULT RAT LIVER SLICES INCUBATED WITH 1-C^{14} OCTANOIC ACID

P values based on analysis of differences between pairs of animals

Mean values ± S.E.

	4 hr. G.H., ad lib.		4 hr. G.H., fasting		12 hr. G.H., fasting		24 hr. G.H., fasting		5 days G.H., ad lib.	
	Control	G.H.	Control	G.H.	Control	G.H.	Control	G.H.	Control	G.H.
No. of animals per group	5	5	5	5	5	5	5	5	5	5
Acetoacetic acid, μg./g. liver	1411 ±78	1449 ±176	1760 ±170	1700 ±190	1530 ±140	1490 ±123	1540 ±400	1680 ±180	1570 ±100	1540 ±90
	P>.05		*P*>.05		*P*>.05		*P*>.05		*P*>.05	
Acetoacetic acid, cts./min./g. liver	7380 ±380	7520 ±420	4870 ±520	5030 ±650	3780 ±750	4630 ±360	5050 ±400	5750 ±600	6640 ±400	6300 ±170
	P>.05		*P*>.05		*P*>.05		*P*>.05		*P*>.05	
Acetoacetic acid, specific activity	5270	5240	2770	2900	3800	3100	3430	3250	4200	4000
$C^{14}O_2$, cts./min./g. liver	10580 ±1600	9590 ±1700	10380 ±920	8720 ±660	—	—	—	—	8800 ±960	11140 ±500
	P>.05		*P*>.05						*P*<.05	

TABLE II

THE EFFECT OF GROWTH HORMONE ON ACETOACETIC ACID AND CO_2 PRODUCTION BY LIVER SLICES FROM ADULT, YOUNG, AND OLD RATS INCUBATED WITH 1-C^{14} OCTANOIC ACID

P values based on analysis of differences between pairs of animals

Mean values ± S.E.

	Adult, fasting 24 hr.		Young, fasting 24 hr.		Young, fed ad lib.		Old, fasting 24 hr.	
	Control	G.H.	Control	G.H.	Control	G.H.	Control	G.H.
No. of animals per group	5	5	8	8	4	4	5	5
Acetoacetic acid, μg./g. liver	1760 ±170	1700 ±190	1540 ±150	1770 ±160	1400 ±120	1650 ±130	1905 ±85	1652 ±60
	$P>.05$		$P<.05$		$P<.05$		$P<.05$	
Acetoacetic acid, cts./min./g. liver	4870 ±520	5030 ±650	4840 ±500	5070 ±600	3970 ±450	3550 ±500	5270 ±360	4440 ±200
	$P>.05$		$P>.05$		$P>.05$		$P=.05$	
Acetoacetic acid, specific activity	2770 — —	2900 — —	3130 ±120	2820 ±150	2830 ±160	2160 ±120	2760 ±160	2770 ±135
			$P=.05$		$P<.05$		$P>.05$	
$C^{14}O_2$, cts./min./g. liver	10380 ±920	8720 ±660	10640 ±540	11880 ±690	7000 ±310	7500 ±520	8800 ±470	8730 ±570
	$P>.05$		$P>.05$		$P>.05$		$P>.05$	

growth hormone (2.5 mg. b.i.d. × 5). In no instance were any statistical differences noted in acetoacetic acid production, incorporation of C^{14} octanoate into acetoacetic acid, or $C^{14}O_2$ production between control and hormone treated animals; however, for incorporation of octanoate carbon into $C^{14}O_2$ in the animals treated for 5 days with growth hormone there appeared to be a significant increase in $C^{14}O_2$ production by slices from treated animals.

In Table II are shown results obtained in young and old rats compared with adult animals. In the young animals fasted 24 hours (to which growth hormone was given at the 20th hour of the fast) in contrast to the adult animals, there was an increased production of acetoacetic acid by liver slices from the hormone treated animals. However, no significant difference was observed in the incorporation of radioactive fatty acid carbon into acetoacetic acid. This, together with the consequent lowering of the specific activity of the acetoacetic acid, suggests that in the young rat the increased amounts of ketone bodies were originating in some non-labelled source. Similar findings, i.e. increased acetoacetic acid production and decreased specific activity of the ketones, were also observed with liver slices from young rats fed ad libitum and injected with growth hormone 4 hours previously.

The results obtained with liver slices from old rats fasted 24 hours and given growth hormone at the 20th hour of the fast contrast with those obtained with slices from both young and adult rats in showing a decreased production of acetoacetic acid together with a decreased incorporation of octanoic acid carbon into acetoacetic acid, which suggests that the effect of growth hormone in the old animal is one of an over-all inhibition of the process of fat catabolism. In none of the age groups was there any difference in $C^{14}O_2$ production between the slices from control and treated animals.

Discussion

From the above data it would appear that growth hormone in the adult rat is not ketogenic and does not increase the rate of catabolism of fatty acid by the liver. These findings are in good agreement with those reported in a recent publication by Allen, Medes, and Weinhouse (1), who used a technique similar to ours but employed radioactive palmitate rather than octanoate. They found no evidence for a ketogenic action of growth hormone or a stimulation of fatty acid utilization by the liver in adult rats.

However, some difference in the action of growth hormone with age of the animal is suggested by the present experiments. Although no ketogenic action was noted in growth hormone treated adult rats, there was a slight increase in acetoacetic acid formation by liver slices from hormone treated young animals and an inhibition in ketogenesis in the elderly rat.

In view of the above data and that of others, it would seem that the ketogenic activity following injections of crude anterior pituitary extracts may not be due to the growth hormone content of these extracts. One must consider that in the preparation of purified growth hormone the ketogenic activity of the extract is sometimes removed. Some purified growth hormone

preparations have been found to be non-ketogenic (16). It is also possible that other pituitary hormones may exert ketogenic effects and hence, be responsible for the activity of crude anterior pituitary extracts. Engel and Engel (7) demonstrated that oxycel corticotropin was highly active in producing ketosis in fasting animals and even in the adrenalectomized animal. Peterson and Lotspeich (14) reported enhanced ketogenesis with thyrotrophic hormone and ascribed the slight ketogenic activity of their growth hormone preparation to its TSH content.

It would thus seem that a ketogenic action and stimulation of fat catabolism cannot be unequivocally ascribed to growth hormone. Hence the concept of growth hormone as an important factor in the catabolism of fat by the liver should perhaps be modified.

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FURTHER STUDIES ON THE ENDOCRINE CONDITIONING OF THE HEPARIN-INDUCED LIPEMIA CLEARING ACTIVITY (LCA) IN THE RAT[1]

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Abstract

The effects of various endocrines and of stress on the "lipemia clearing activity" (LCA) which is elicited in rat plasma by intravenous heparin injection were studied.

High dosage cortisone accelerated LCA, but low dosage cortisone did not affect it. Severe catabolic stress accelerated LCA. Application of a single mild stress was ineffective, but the combination of two mild stressors accelerated LCA. ACTH had no influence on LCA, but adrenalectomy accelerated it. Stilbestrol inhibited LCA at both a low and a high dosage level, but there was no difference in LCA production between males and females or between estrous and diestrous females. Estrous females, however, displayed less LCA than ovariectomized females. Anterior pituitary extract inhibited LCA in females and hypophysectomy accelerated it in both sexes. A synthetic oil emulsion yielded the same qualitative information as lipemic plasma wherever it was used as a substrate for the clearing activity of postheparin plasma.

Thus, with the exception of the effects of sex difference and low dosage cortisone, most of the previously reported endocrine effects on the LCA that follows subcutaneous heparin were also obtained when heparin was injected intravenously. The physiological significance of these findings is discussed.

Introduction

In a previous publication (1), certain endocrine effects on the lipemia clearing activity (LCA) induced by subcutaneous heparin injection were reported. In that study LCA was assayed by recording the late phase of the clearing of postheparin – lipemic plasma mixtures, i.e. at 20 and 40 minutes after mixing. Under those conditions it was found that LCA is inhibited by estradiol, low dosage glucocorticoids, thyroxine, and anterior pituitary extract, accelerated by high dosage glucocorticoids and hypophysectomy, and not affected by ACTH or mild cold exposure. Similar actions on LCA of low dosage glucocorticoids and of thyroxine have been reported by other workers (5, 7).

Since several of the above findings might be due to endocrine effects on the absorption of the subcutaneously injected heparin, and since it has been recently postulated (3) that only the early period of physical clearing is directly proportional to the lipolytic process in postheparin – lipemic plasma mixtures, it was considered necessary to reinvestigate the principal endocrine effects on LCA, using intravenous heparin and recording clearing at 5, 10, and 20 minutes after mixing.

In addition, further attempts were made to analyze the relationship between the effects on LCA of injected cortisone and stress, as well as to determine the physiological or pharmacological nature of the effects of injected estrogen on LCA.

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Contribution from the Anatomy Department, University of British Columbia, Vancouver, B.C.

Materials and Methods

The same general experimental arrangement as in a previous study (1) was employed: heparin was injected into control rats and experimental rats subjected to various endocrine pretreatments. The citrated plasma of these heparin-injected animals was then mixed with citrated lipemic plasma obtained from suitable donors, and the turbidity loss ("clearing") of the mixtures at any given time was expressed as percentage of the initial turbidity at mixing. The procedures for sampling and citrating of plasma, procurement of lipemic plasma, and turbidimetry were the same as were used previously.

The following specific modifications of our previous technique were adopted:

(1) Heparin (Connaught Laboratories) was injected intravenously into the tail vein of the rats, 15 minutes before their plasma was sampled, at the dosage level of 0.2 mg./kg. Every animal received its heparin dose in 0.3 ml. of saline. The injections were given with a tuberculin syringe and a 25 gauge needle.

(2) The turbidity of postheparin – lipemic plasma mixtures was determined immediately and 5, 10, and 20 minutes after mixing.

(3) All clearing systems were incubated at 37° C.

The animals were injected with heparin, and their plasmata sampled and assayed for LCA in successive pairs consisting of one control and one experimental, in accordance with a strictly timed, staggered schedule.

Since control and experimental plasmata were run in pairs against the same lipemic plasma batch and since the initial turbidity of the lipemic plasma varied from batch to batch, the results were analyzed statistically with the null hypothesis (8). Thus, in every experiment, the mean difference $\pm\epsilon$ between the clearing of every experimental and its corresponding control mixture was computed for each time interval. In the tabulation of these differences, the clearing values of the controls were arbitrarily taken as positive references against which those of the experimentals were compared. Thus, a mean difference with a negative sign implies that the experimental plasmata displayed less LCA than the control plasmata, whereas a positive sign indicates the opposite.

As in the past, several morphological end points of hormone action or withdrawal were recorded in all experiments. These included body weight changes and left adrenal weight in most experiments, daily vaginal smears and uterine weights in the estrogen, ovariectomy, and estrous cycle experiments, and ovarian and seminal vesicle weights in the hypophysectomy and pituitary extract experiments. Animals in estrus and diestrus were selected after the typing of their daily vaginal smears for several cycles. Of these collateral data, only the body weight changes and the left adrenal weights were tabulated. Since the other findings merely confirmed the specific biological effectiveness of the endocrine treatments, they have been omitted from presentation.

In three instances, LCA was assayed against both lipemic plasma and an oil emulsion substrate. These were the experiments in which the effects on

LCA of low dosage cortisone, 72 hours fasting, and stilbestrol were tested. In the case of low dosage cortisone, two different experiments were performed, one using oil emulsion, the other using lipemic plasma as substrate. In the case of fasting and stilbestrol, all postheparin plasmata were divided into two lots, one of which was assayed against oil emulsion, the other against lipemic plasma.

The oil emulsion consisted of 10% peanut oil stabilized with 0.5% Tween 60. This emulsion was diluted 1:10 with distilled water just before use. The technique of LCA assay was identical for the two substrates except that the postheparin plasma was not citrated when mixed with the oil emulsion.

All hormones were injected subcutaneously.

The dosages, durations, and other particulars of the experimental treatments have been summarized in the tables containing the results.

Results

(1) *Cortisone* (*Table I*)

Catabolic, high dosage cortisone accelerated LCA (expt. No. 1), but noncatabolic, low dosage cortisone had no significant effect on the clearing of either lipemic plasma (expt. No. 2) or oil emulsion (expt. No. 3). When, however, the mild stress of 20 hours' fasting was combined with low dosage cortisone, a borderline acceleration of clearing occurred, along with significant catabolism (expt. No. 4).

TABLE I

EFFECTS OF CORTISONE ON LCA (MALE RATS)

Group	No. animals	Mean % body wt. change during expt. ± ϵ	Adrenal wt. (mg./100 g. terminal BW) ± ϵ	Mean clearing difference of experimentals from controls ± ϵ		
				5 min. after mixing	10 min. after mixing	20 min. after mixing
(1) Cortisone, 10 mg./kg./rat/day, 1 week						
Controls	6	+14.2 ± 2.3	7.0 ± 0.37			
Cortisone	6	−29.7 ± 0.9	4.5 ± 0.47	+18.4 ± 3.6 $p < 0.01$	+26.4 ± 4.2 $p < 0.01$	+19.2 ± 5.3 $p = 0.02$–0.01
(2) Cortisone, 1 mg./kg./rat/day, 1 week						
Controls	6	+15.2 ± 4.9	6.8 ± 0.90			
Cortisone	6	+18.1 ± 2.7	6.5 ± 0.30	+1.1 ± 0.7 $p = 0.2$–0.1	−0.3 ± 1.6 $p = 0.9$–0.8	−0.3 ± 1.6 $p = 0.9$–0.8
(3) Cortisone, 1 mg./kg./rat/day, 1 week, oil emulsion substrate						
Controls	5	+19.9 ± 1.3	—			
Cortisone	5	+15.1 ± 7.0	—	−3.3 ± 3.2 $p = 0.4$–0.3	−4.2 ± 3.8 $p = 0.4$–0.3	−1.8 ± 2.8 $p = 0.6$–0.5
(4) Cortisone, 1 mg./kg./rat/day, 1 week, plus 20 hours fasting preterminally						
Controls	6	+ 5.8 ± 2.3	5.0 ± 1.05			
Cortisone	6	−10.2 ± 1.7	4.8 ± 0.03	+6.6 ± 2.9 $p = 0.1$–0.05	+12.2 ± 5.7 $p = 0.1$–0.05	+15.2 ± 6.7 $p = 0.1$–0.05

(2) *Stress* (*Table II*)

To exclude specific effects, three different stressors were studied, namely, fasting, cold, and fracture (under ether anesthesia).

Moderate and severe fasting, lasting 48 hours or longer and associated with significant catabolism and adrenal hypertrophy, greatly accelerated the clearing of both lipemic plasma and oil emulsion (expts. Nos. 1, 2, 3, 4), but a mild fast of 24 hours did not affect LCA (expt. No. 5).

TABLE II

EFFECTS OF STRESS ON LCA (MALE RATS)

Group	No. animals	Mean % body wt. change during expt. ± ϵ	Adrenal wt. (mg./100 g. terminal BW) ± ϵ	Mean clearing difference of experimentals from controls ± ϵ		
				5 min. after mixing	10 min. after mixing	20 min. after mixing
(1) Severe fasting, 72 hours						
Controls	8	+ 0.7 ± 2.5	8.2 ± 0.59			
Fasting	8	−25.1 ± 1.1	11.6 ± 1.25	+19.5 ± 4.2 $p < 0.01$	+25.6 ± 5.1 $p < 0.01$	+25.3 ± 6.7 $p < 0.01$
(2) Severe fasting, 72 hours, oil emulsion substrate						
Controls	6	+ 0.7 ± 2.5	—			
Fasting	6	−24.5 ± 1.5	—	+16.1 ± 2.8 $p < 0.01$	+22.0 ± 4.0 $p < 0.01$	+20.7 ± 6.5 $p = 0.02$
(3) Severe fasting, 64 hours						
Controls	5	+ 7.2 ± 1.4	—			
Fasting	5	−31.5 ± 0.2	—	+22.2 ± 4.9 $p = 0.01$	+24.8 ± 6.8 $p = 0.02$	+18.3 ± 8.8 $p = 0.2$–0.1
(4) Moderate fasting, 48 hours						
Controls	9	+ 2.2 ± 0.8	6.5 ± 0.50			
Fasting	9	−21.4 ± 1.4	9.4 ± 0.79	+ 9.3 ± 2.7 $p < 0.01$	+13.0 ± 3.7 $p < 0.01$	+13.6 ± 3.7 $p < 0.01$
(5) Mild fasting, 24 hours						
Controls	7	+ 2.2 ± 0.8	8.7 ± 0.40			
Fasting	7	− 5.7 ± 0.7	8.4 ± 0.50	− 0.6 ± 1.9 $p = 0.8$–0.7	− 2.3 ± 2.4 $p = 0.4$–0.3	+ 0.4 ± 2.9 $p = 0.9$–0.8
(6) Severe cold exposure, 0–2 C.°, 5 days						
Controls	6	+ 5.6 ± 1.8	8.0 ± 0.59			
Cold exposure	6	− 5.0 ± 1.1	11.0 ± 0.51	+11.2 ± 2.7 $p < 0.01$	+18.0 ± 3.8 $p < 0.01$	+21.9 ± 4.0 $p < 0.01$
(7) Mild cold exposure, 2–5 C.°, 24 hours						
Controls	6	+ 1.3 ± 0.7	7.2 ± 0.71			
Cold exposure	6	− 5.3 ± 1.5	8.5 ± 0.80	+ 1.9 ± 3.8 $p = 0.7$–0.6	+ 5.2 ± 4.8 $p = 0.4$–0.3	+ 4.7 ± 5.1 $p = 0.4$
(8) Combined mild cold exposure and fasting, 24 hours						
Controls	6	+ 2.6 ± 0.9	8.1 ± 0.24			
Cold and fasting	6	−15.1 ± 0.6	9.9 ± 0.57	+16.9 ± 2.4 $p < 0.01$	+27.0 ± 4.5 $p < 0.01$	+28.4 ± 4.9 $p < 0.01$
(9) Fracture, both hind legs within 24 hours						
Controls	6	+ 3.4 ± 1.3	7.5 ± 0.28			
Fracture	6	− 2.1 ± 0.5	8.2 ± 0.43	+ 4.1 ± 2.1 $p = 0.1$–0.05	+ 7.3 ± 2.9 $p = 0.1$–0.05	+12.3 ± 4.7 $p = 0.05$

Similarly, severe cold exposure for 5 days, associated with appreciable catabolism and adrenal enlargement, accelerated LCA (expt. No. 6), whereas mild cold exposure for 24 hours had no effect on it (expt. No. 7).

When two mild stressors which were ineffective alone (24 hours' fasting and 24 hours' cold exposure) were combined, a marked acceleration of clearing occurred, along with potentiated catabolism and adrenal hypertrophy (expt. No. 8).

Fracture of the hind legs (within the short period studied) caused a borderline enhancement of clearing, along with little catabolism and insignificant adrenal hypertrophy (expt. No. 9).

(*3*) *ACTH and Adrenalectomy* (*Table III*)

Large amounts of two different brands of ACTH (of which, however, only one elicited an adrenal weight response) failed to affect LCA (expts. Nos. 1, 2). Adrenalectomy caused a slight acceleration of a borderline significance (expt. No. 3).

TABLE III

EFFECTS OF ACTH AND ADRENALECTOMY ON LCA (MALE RATS)

Group	No. animals	Mean % body wt. change during expt. ± ϵ	Adrenal wt. (mg./100 g. terminal BW) ± ϵ	Mean clearing difference of experimentals from controls ± ϵ		
				5 min. after mixing	10 min. after mixing	20 min. after mixing
(1) ACTH, Ciba, 3 × 2 mg./rat/day, 2 days						
Controls	6	+ 0.3 ± 0.7	7.2 ± 0.33			
ACTH	6	+ 2.3 ± 1.7	8.8 ± 0.14	− 1.3 ± 1.9 p =0.6–0.5	− 2.8 ± 3.3 p =0.5–0.4	− 4.8 ± 5.2 p =0.4–0.3
(2) ACTH, Nordic Biochemicals, 3 × 2 mg./rat/day, 2 days						
Controls	6	− 0.4 ± 2.9	7.6 ± 0.40			
ACTH	6	+ 3.2 ± 1.7	7.9 ± 0.59	+1.0 ± 1.7 p =0.6–0.5	+ 1.7 ± 2.9 p =0.6–0.5	+ 3.3 ± 4.2 p =0.5–0.4
(3) Adrenalectomy, 10 days						
Controls	6	+16.4 ± 1.9	—			
Adrenalectomy	6	+16.8 ± 2.6	—	+ 6.9 ± 2.3 p =0.05–0.02	+11.4 ± 5.9 p =0.1	+15.1 ± 5.5 p =0.05–0.02

(*4*) *Estrogen* (*Table IV*)

Injected estrogen had a pronounced inhibitory effect on LCA (expts. Nos. 1, 2). This inhibition was exerted despite the induction of catabolism and adrenal hypertrophy, regardless of dosage level, and against either lipemic plasma or oil emulsion substrate.

There was no significant difference between the LCA of males and females (expt. No. 3) or between estrous and diestrous females (expt. No. 4). Estrous females, however, displayed significantly less LCA than ovariectomized females (expt. No. 5).

(5) *Anterior Pituitary Extract and Hypophysectomy (Table V)*

Anterior pituitary extract inhibited LCA in the female while simultaneously causing catabolism and adrenal enlargement (expt. No. 1).

Hypophysectomy accelerated LCA markedly in females (expt. No. 2) as well as in males (expt. No. 3) and it produced catabolism and adrenal atrophy in both sexes.

TABLE IV

EFFECTS OF ESTROGEN ON LCA (FEMALE RATS, EXCEPT NO. 3)

Group	No. animals	Mean % body wt. change during expt. ± ϵ	Adrenal wt. (mg./100 g. terminal BW) ± ϵ	Mean clearing difference of experimentals from controls ± ϵ		
				5 min. after mixing	10 min. after mixing	20 min. after mixing
(1) Stilbestrol, low (0.25 mg./rat/day) and high (4 mg./rat/day) dosage, 2 weeks						
Controls	6	+19.6 ± 3.0	14.1 ± 0.7			
Stilb. low dosage	6	−15.7 ± 1.5	23.8 ± 3.2	− 8.2 ± 1.4 $p < 0.01$	−16.4 ± 2.2 $p < 0.01$	−26.6 ± 2.9 $p < 0.01$
Stilb. high dosage	6	−12.0 ± 0.7	23.6 ± 2.2	− 6.7 ± 2.2 $p = 0.05$–0.02	−13.8 ± 3.5 $p = 0.01$	−22.3 ± 5.2 $p < 0.01$
(2) Stilbestrol, same expt. as above, oil emulsion substrate						
Stilb. low dosage	6	—	—	− 4.0 ± 1.2 $p = 0.02$	− 7.1 ± 1.8 $p = 0.02$–0.01	−15.1 ± 2.2 $p < 0.01$
Stilb. high dosage	6	—	—	− 2.2 ± 1.2 $p = 0.2$–0.1	− 3.9 ± 2.9 $p = 0.4$–0.3	− 9.4 ± 3.7 $p = 0.05$
(3) Sex difference						
Males (control)	6	—	—			
Females	6	—	—	+ 4.0 ± 1.6 $p = 0.1$–0.05	+ 3.3 ± 1.9 $p = 0.2$–0.1	+ 2.1 ± 1.1 $p = 0.2$–0.1
(4) Diestrus versus estrus						
Diestrus (control)	8	+ 5.2 ± 0.2	—			
Estrus	8	+ 5.8 ± 1.8	—	+ 4.0 ± 3.0 $p = 0.3$–0.2	+ 4.6 ± 4.2 $p = 0.4$–0.3	+ 1.5 ± 4.4 $p = 0.8$–0.7
(5) Ovariectomy (2 weeks) versus estrus						
Ovariect. (control)	9	+11.5 ± 1.8	—			
Estrus	9	+ 8.7 ± 1.7	—	− 8.4 ± 1.7 $p < 0.01$	−12.6 ± 2.9 $p < 0.01$	−15.4 ± 3.4 $p < 0.01$

TABLE V

EFFECTS OF ANTERIOR PITUITARY EXTRACT AND HYPOPHYSECTOMY ON LCA (FEMALES, EXCEPT NO. 3)

Group	No. animals	Mean % body wt. change during expt. ± ϵ	Adrenal wt. (mg./100 g. terminal BW) ± ϵ	Mean clearing difference of experimentals from controls ± ϵ		
				5 min. after mixing	10 min. after mixing	20 min. after mixing
(1) Whole beef anterior pituitary extract, 2 × 0.1 g./rat/day, 5 days (in females)						
Control	9	+ 6.3 ± 3.8	14.8 ± 0.8			
Ant. pituit. extract	9	− 4.9 ± 2.1	25.5 ± 1.7	−4.45 ± 1.6 $p = 0.05$–0.02	− 8.3 ± 3.1 $p = 0.05$–0.02	−12.8 ± 3.5 $p < 0.01$
(2) Hypophysectomy, 3 weeks (in females)						
Controls	6	+25.8 ± 9.5	14.4 ± 0.5			
Hypophysectomy	6	− 6.6 ± 1.7	5.2 ± 0.1	+22.6 ± 5.4 $p < 0.01$	+23.2 ± 4.1 $p < 0.01$	+14.8 ± 2.9 $p < 0.01$
(3) Hypophysectomy, 3 weeks (in males)						
Controls	7	+50.6 ± 3.7	7.9 ± 0.4			
Hypophysectomy	7	− 5.7 ± 1.6	3.8 ± 0.2	+27.4 ± 2.0 $p < 0.01$	+25.4 ± 2.5 $p < 0.01$	+20.5 ± 6.5 $p = 0.02$–0.01

Discussion

The data of the present study show that, with two exceptions, most of the previously reported (1) endocrine effects on the LCA which follows subcutaneous heparin administration can also be obtained if heparin is injected intravenously. The two exceptions were the sex difference and the inhibitory action of low dosage cortisone, which could not be repeated with the intravenous technique.

It is, therefore, possible that the previous finding of a sex difference was not a true endocrine effect but the result of modified absorption or partial inactivation of the subcutaneously injected heparin. In the case of cortisone, however, the discrepancy between results with the two techniques was less marked, suggesting the existence of a true qualitative difference between the actions of low and high dosage cortisone, regardless of the route of heparin administration. Thus, whereas large amounts of cortisone greatly accelerated the LCA that followed either subcutaneous or intravenous heparin injection, small amounts of this steroid inhibited the LCA that followed subcutaneous heparin and failed to affect the LCA that followed intravenous heparin. It is interesting that even with the subcutaneous heparin technique, the inhibitory effect of low dosage glucocorticoids had been found to disappear upon prolonged treatment (1).

In the light of these new data, it is necessary to revise our previous interpretation of the relationships of postheparin LCA to the adrenal cortex or the female gonad.

If one accepts as true endocrine effects only the LCA changes obtained after intravenous heparin, it would appear that there is only one action of injected cortisone, namely acceleration of LCA, and that this action can be obtained only with large, catabolic amounts of this steroid. Severe, catabolic stress imitates the effects of large amounts of injected cortisone, whereas mild stress or small amounts of injected cortisone are both ineffective. The present data also suggest that there may be a synergism between cortisone and stress with regard to LCA acceleration, much in the manner of the synergism that has been demonstrated with regard to protein catabolism or thymicolymphatic involution, and led to the "permissive" (6) or catalytic concept of glucocorticoid function. It remains to be shown by future work whether stress acts on LCA through the adrenal or whether stress and high dosage injected cortisone act through independent mechanisms. Since the availability of protein has been shown to accelerate LCA (2, 4) (by providing an acceptor for the fatty acids released), it is possible that both stress and injected high dosage cortisone increase LCA because they provoke marked protein catabolism.

So far, attempts at finding out whether stress acts through the adrenal have failed because all adrenalectomized animals that were exposed to severe stress died before their plasma could be sampled. A further obstacle to the unitarian interpretation of the stress and cortisone data can be found in the LCA-accelerating effect of adrenalectomy and hypophysectomy. Although

the effect of adrenalectomy is slight and of a borderline significance, it has been obtained consistently, like the more pronounced and highly significant effect of hypophysectomy.

At the moment, the most prominent common feature of high dosage cortisone, stress, hypophysectomy, and adrenalectomy seems to be the induction of protein catabolism. Further investigations will have to decide whether all four treatments accelerate LCA through this non-specific common denominator.

Directly opposed to the above accelerator effects are the actions of injected estrogen and anterior pituitary extract, which inhibit LCA despite the induction of catabolism and adrenal hypertrophy. The inhibitory effect of injected estrogen was obtained with the natural as well as with the synthetic hormone, and it proved independent of the route of heparin administration. Since, however, intravenous heparin elicited the same LCA in males and females or in estrous and diestrous females, it appears that physiological differences in endogenous plasma estrogen levels are not sufficiently great to influence postheparin LCA. Furthermore, the observation that hypophysectomy accelerates LCA in both sexes would indicate that its effect is not mediated through ovarian underfunction, as assumed originally. On the other hand, the finding that estrous females displayed less LCA than ovariectomized females suggests that extreme differences in endogenous plasma estrogen levels may affect LCA significantly. Thus, the final settlement of the physiological or pharmacological nature of the inhibitory action of injected estrogen requires additional research.

Since, in the three typical instances in which it was used as a substrate for clearing, the oil emulsion yielded the same qualitative information as lipemic plasma, its use in LCA studies is further endorsed. This is evidently of particular value for clinical LCA investigations where, in view of the great difficulty of testing simultaneously pairs of control and experimental individuals, only substrates of repeatable composition and turbidity (such as synthetic oil emulsions) are practical.

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THE CONVERSION OF FAT TO CARBOHYDRATE DURING EMBRYONATION OF ASCARIS EGGS[1]

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Abstract

While ascaris eggs developed to the vermiform stage (10 days) both lipids and carbohydrates (glycogen and trehalose) decreased in amount. During the next 15 days, in which the embryo became infective, lipids continued to decrease, but at a greater rate, whereas carbohydrate was completely resynthesized. Examination of the possible sources of the required carbon revealed that protein, non-protein nitrogenous compounds, phosphorus compounds, metabolic acids, glycerol, and volatile acids occurring in the triglycerides were not adequate and that carbon dioxide was not extensively fixed. Direct evidence for the conversion of triglyceride acids to carbohydrate was furnished by the increase in lipid-free dry weight, which corresponded closely with the increase in carbohydrate. Moreover, the amount of lipid carbon which disappeared was equal to the sum of the carbon dioxide and carbohydrate carbon which appeared, and oxygen consumption was insufficient to account for the complete combustion of lipid carbon. It was concluded that the carbon of partially oxidized fragments of fatty acids, possibly acetylcoenzyme A, was incorporated into glycogen and trehalose, and hence that ascaris eggs in this stage of their development were able to bring about a net conversion of fat to carbohydrate.

Introduction

A previous report from this laboratory (12) described the lipids of ascaris eggs and some of the changes which occurred in them in the course of embryonation. The major lipids present were ascaryl alcohol, phospholipids, and a triglyceride fraction. Of these, ascaryl alcohol did not change in amount during development, since it is concerned primarily in the structure of the vitelline membrane (14). Phospholipids were also fairly constant, but the abundant triglyceride fraction was used extensively throughout the whole developmental period. This fraction contained major amounts of esterified non-volatile acids averaging 18–20 carbons (mean molecular weight, 296) and minor amounts of volatile acids belonging to the C_2—C_6 series. In a later communication (15) it was shown that the egg also contained a considerable concentration of carbohydrates, which were found to consist almost entirely of glycogen and trehalose in approximately equal amounts.

The present investigation revealed an interesting relationship between the fats and the carbohydrates. From the 11th to the 25th developmental day, lipids decreased at a maximum rate, whereas carbohydrate, which had previously been used extensively in the course of energy metabolism, was completely resynthesized.

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An analysis of the various sources which might provide carbon for carbohydrate synthesis by the egg led to the conclusion that most of the required carbon must come from the non-volatile triglyceride acids. As this conclusion does not appear to need qualification in any important respect, the results of the investigation provide the first clear evidence for the conversion of fat to carbohydrate in an animal tissue.

Methods

Uterine eggs were collected from a number of mature females (12). In each experiment, with specific exceptions, 2 g. portions taken from a large and well-mixed batch of eggs were *decoated* (12) and set aside at 3° C. (unembryonated eggs) or placed in flasks in the presence of 1% sodium carbonate and shaken continuously at 30° C. for appropriate intervals. The concentration of alkaline salt used was sufficient to reduce microbial growth to very low levels, while leaving the eggs unaffected. Under these conditions at least 95% of the embryos matured uniformly. Unembryonated eggs, or eggs in various stages of development, could be stored at 3° C. for several weeks without change in composition or loss of viability. In experiments in which samples were withdrawn periodically from the incubator, advantage was taken of this stability in order to carry out simultaneous analyses. In general, 2 g. samples provided sufficient material for unequivocal analysis. In a few experiments, however, larger amounts were used.

Fresh weights were recorded in terms of *uterine eggs*, and dry weights in terms of *decoated, unembryonated egg solids*, in accordance with previous practice (12). As a result, the percentage composition of embryonated or partially embryonated eggs as given in the tables does not represent the actual percentage composition, which will usually be somewhat higher owing to the disappearance of food reserves. This discrepancy is of no importance in the interpretation of the results.

Before analysis, the eggs were killed by being heated for 5–10 minutes in a boiling water bath, and then homogenized in an ice bath, using a stainless steel pestle. Several minutes of homogenization were needed to break up 95% of the shells and embryos. Each sample was checked microscopically, as large molecules like glycogen could not be extracted through the unbroken shell. The homogenate was diluted to about 10 ml., and portions for analysis were withdrawn with wide orifice (serological) pipettes. The standard deviation from the mean of the dry weights of six 2 ml. samples withdrawn in this way from a single homogenate was about $\pm 1\%$.

In a typical experiment, the dry weight of the eggs was determined on approximately half of the homogenate by evaporation *in vacuo* over calcium chloride at room temperature. Lipids were extracted from the residue with three changes of chloroform–methanol (2:1, v/v) over a period of 24 hours at room temperature. These extracts were then washed with water (18) to remove non-lipid extractives, and the lipids dried and weighed. As considerable non-lipid matter appeared in the unwashed lipid extracts, lipid-free

dry weight could not be determined directly, but instead was estimated as the difference between total dry weight and lipid weight.

Portions of the homogenate were taken for the determination of alkali-stable carbohydrate (glycogen and trehalose) (15) by the anthrone method as described by Mokrasch (27). At least 95% of the total carbohydrate was alkali-stable. Glycogen was isolated from another portion of the alkaline digest after addition of alcohol (15) and determined by the same method. Trehalose was estimated as the difference between alkali-stable carbohydrate and glycogen, because direct analysis of the ethanol-containing supernatant solution gave high results. The reason for this interference by ethanol lies in the observation of Kahan (21) that the amount of water added with the sample to the anthrone reagent must not vary outside narrow limits.

Nitrogen was determined by nesslerization (23) following Kjeldahl digestion (25). Protein nitrogen was precipitated by addition to the homogenate of an equal volume of cold, 10% trichloroacetic acid; non-protein nitrogen (including polypeptides) remained in the supernatant solution. The same precipitation technique was used to separate acid-soluble and acid-insoluble phosphorus-containing compounds, which were determined according to Fiske and Subbarow (17). The phosphorus content of the lipids was also determined.

In one experiment (Table VII) glycerol and volatile acids were determined in the lipids. For this purpose the lipids were saponified in barium hydroxide (9) and insoluble barium salts of the higher fatty acids were separated, washed, and discarded, since the more unsaturated acids were badly oxidized during saponification. The barium sulphate precipitated by neutralization of the supernatant solution was also washed and discarded. Volatile acids were then distilled from the acidified solution, neutralized, dried, extracted into chloroform–butanol (99:1, v/v) (13) and titrated. Barium sulphate was removed from the neutralized distillation residue as described above, and the concentrated solution was chromatographed on thick paper (Eaton-Dikeman, No. 627-030) in order to purify the glycerol, which was then eluted from the paper and determined by periodate oxidation (29).

Carbon-14 distribution in the glucose derived from trehalose and glycogen fractions following 4 hours' hydrolysis in N H_2SO_4 was determined according to Bernstein *et al.* (2) with some modification. The acid hydrolyzate was neutralized with barium hydroxide, after which the glucose was chromatographed on thick paper (see above) and eluted. The radioactive eluates were diluted with pure glucose to a final concentration of 1 millimole (mM.) in 10 ml. This solution was fermented with *Leuconostoc mesenteroides* (Strain 39, American Type Culture Collection No. 12291) to carbon dioxide, ethanol, and lactic acid. Carbon dioxide (carbon 1) was collected in alkali during the fermentation. Ethanol (carbons 2 and 3) was purified by distillation, oxidized to acetic acid (2), and the acetic acid purified by steam distillation and chromatography on silica gel (26). An aqueous solution of the acetate was degraded by the Schmidt reaction (10, 33) to carbon dioxide (carbon 3)

and methylamine. From the latter, carbon dioxide (carbon 2) was obtained by permanganate oxidation in a sealed tube (10, 33). The residue remaining after distillation of the ethanol, above, was made alkaline to phenolphthalein and extracted continuously with ether. This extract was discarded, the residue was acidified, and lactic acid was extracted into ether and then into water following evaporation of the ether. Lactic acid was determined on a portion of the aqueous solution according to Barker and Summerson (1). The remainder was oxidized with chromium trioxide (7) to carbon dioxide (carbon 4) and acetic acid, which was purified as described above and then oxidized by wet combustion (10, 39) (carbons 5 and 6). Carbon dioxide was determined by back-titration following distillation *in vacuo* into barium hydroxide solution (39). The barium carbonate was then plated and the C^{14} content determined (10).

Results

The premise that ascaris eggs under normal physiological conditions are permeable only to gases and water vapor (14, 32, 38) is fundamental to the interpretation of the experimental results. Good examples of the complete retention of metabolites are found in the analytical data included in Tables I and III, and eggs were always embryonated in a non-nutrient medium. Most experiments covered a 25 day incubation period. The embryo required 10 days to become vermiform and another 10 days to molt once and become infective. An additional five days was necessary, however, for changes in carbohydrates to reach completion. During the first 10 days the respiratory rate increased to a maximum, after which it decreased markedly (32). Most of the phenomena to be reported occurred during the period of declining respiration.

Nitrogenous Compounds

The results of several experiments led to the conclusion, not only that total nitrogen remained constant during embryonation, but also that the relative proportions of protein and non-protein nitrogen remained unchanged.

TABLE I

NITROGEN IN EMBRYONATING EGGS

	Days					
	0	5	10	15	20	25
	N, g. per 100 g. decoated, unembryonated egg solids					
Total N*	6.6	6.8	6.5	6.5	6.5	6.6
Protein N†	5.9	6.0	5.8	5.8	5.8	5.8
NPN	0.72	0.75	0.66	0.71	0.71	0.76

*In this experiment total N was calculated as the sum of protein-N and NPN. Several other experiments in which total N was determined directly after 0, 10, and 25 days gave the same average value.

†Protein N includes relatively small amounts of lipid N, nucleoprotein N, and chitin N.

In the experiment summarized in Table I, 2-g. samples of uterine eggs were embryonated for periods up to 25 days, duplicate samples being removed at 5-day intervals. No important change was observed in total, protein, or non-protein nitrogen.

Fairbairn and Passey (15) reported that the shell of infective eggs accounted for 39% of the egg volume. It was of interest, therefore, to determine the distribution of nitrogen between shell and vitellus (including the perivitelline fluid). The procedure finally adopted was best suited to unembryonated eggs, but could also be used (less accurately) with 10-day eggs. No method for separating shells from infective embryos was found. There is no reason to believe, however, that any significant exchange of nitrogen between shell and embryo occurred during development, inasmuch as the shell and vitelline membrane are completely formed following fertilization of the egg in the uterus (16, 43), i.e. before embryonation begins. Two-gram egg samples were lightly homogenized and centrifuged. The turbid supernate was filtered through bolting silk with gentle suction and the residue was again homogenized and centrifuged. This process was repeated until the shell and protoplasmic fractions appeared, by microscopic examination, to be well separated. The results of one such experiment are summarized in Table II. As expected, the shell fraction contained little non-protein nitrogen. Protein nitrogen in the protoplasm was 3.1%, which is equivalent to 19.4% protein if the conventional conversion factor (6.25) is applied. Advantage will be taken of this estimate subsequently.

During their development cleidoic eggs generally accumulate waste (non-protein) nitrogen in the form of insoluble uric acid (28). There was a possibility, therefore, that in ascaris eggs the non-protein nitrogen experimentally determined (Table I) represented soluble nitrogen only, and that *accumulation* of non-protein nitrogen was disguised by the appearance of insoluble nitrogen in the protein fraction, where it would be recorded as protein nitrogen. The shell and protoplasm fractions obtained from unembryonated and 10-day eggs were therefore extracted with lithium carbonate (5) in order to dissolve any uric acid which was present, and the extract was analyzed (4). Traces of uric acid were found in the protoplasm of 10-day eggs (9 mg. per 100 g. of unembryonated egg solids), but not in unembryonated eggs. The

TABLE II

NITROGEN DISTRIBUTION IN THE SHELL AND VITELLUS OF UNEMBRYONATED EGGS

	Dry weight	Total N	Protein N*	NPN
	g. per 100 g. of decoated, unembryonated egg solids			
Vitellus	63 ± 3.0†	3.7	3.1	0.6
Shell	36	2.9	2.8	0.08

*N-Acetylglucosamine from chitin contributes an undetermined amount of nitrogen to the shell fraction.

†Standard deviation of five samples.

extracts showed only a minor absorption in the 240–290 mμ region. Evidently, uric acid and other insoluble purines were not present in important amounts. In another experiment, a more general procedure was applied in order to detect any insoluble non-protein nitrogen. Intact 10- and 25-day eggs were extracted with a little hot water, which destroys the semipermeability of the egg by melting the vitelline membrane (14). Small molecules, e.g. trehalose (15), then pass freely through the residual chitinous shell. The suspension was centrifuged, and trehalose and non-protein nitrogen were determined in the extract. If highly insoluble non-protein nitrogen were present in the eggs, then in succeeding extracts the ratio NPN: trehalose should rise consistently. Actually, however, the ratio remained constant, with most of the trehalose and NPN appearing in the first extract. There is no reason to doubt, therefore, that the values for NPN shown in Table I are accurate and that NPN changed little if any during embryonation.

It is conceivable that extensive qualitative changes could occur in the egg protein without notable changes in protein nitrogen. Accumulation in the proteins of glutamine or asparagine, for example, would provide a method for binding nitrogen which might otherwise appear as non-protein nitrogen. This possibility was tested by observing the pattern of amino acids obtained by paper chromatography of the hydrolyzed protein fractions (34) prepared from 0-, 5-, 10-, 15-, 20-, and 25-day eggs. As no notable differences were seen in the amino acids of these preparations either in kind or amount, it seemed certain that nitrogen did not accumulate in the form of nitrogen-rich amino acids.

Phosphorus

Phosphorus distribution was examined in two experiments, which were similar in design to those described for nitrogen distribution. Total, acid-soluble, inorganic, and lipid phosphorus were determined directly. Organic

TABLE III

PHOSPHORUS IN EMBRYONATING EGGS*

	Days					
	0	5	10	15	20	25
	P, g. per 100 g. decoated, unembryonated egg solids					
Total P	0.60	0.59	0.60	0.63	0.61	0.62
Acid-soluble P	0.37	0.36	0.24	0.28	0.29	0.29
Inorganic	0.11	0.08	0.07	0.07	0.06	0.06
Organic†	0.26	0.28	0.17	0.21	0.23	0.23
Acid-insoluble P†	0.23	0.23	0.36	0.35	0.32	0.33
Lipid	0.15	0.14	0.15	0.14	0.12	0.12
Nucleic acid plus protein†	0.08	0.09	0.21	0.21	0.20	0.20

*Average values from two experiments.
†Values derived by difference.

acid-soluble phosphorus, and nucleic acid plus protein phosphorus, were estimated by difference. The greater part of the total phosphorus in unembryonated eggs was present in the organic, acid-soluble fraction (43%) and in the phospholipids (25%) (Table III). The value of the latter was about 30% higher than the figure previously reported by Fairbairn (12), who determined phosphorus on the isolated phospholipids and who may, therefore, have encountered losses. During embryonation the greatest change observed took place in the organic acid-soluble fraction during the first 10 days. This decrease was reflected for the most part in an increase in the nucleic acid and phosphoprotein fraction, as might be expected since this is the period of maximum cell-division. Changes in other fractions were small.

Dry Weight, Lipids, Carbohydrates

The values recorded in Table IV are averages derived from the analysis of duplicate egg samples in at least three experiments. A continual decrease in *total* dry weight was observed, which was, however, less pronounced between 10–25 days than 0–10 days. The lipids, on the other hand, disappeared much faster in the second period than in the first. This apparent anomaly was resolved by the behavior of the carbohydrates, whose concentration was reduced to 56% of the initial value by the end of 10 days, but was completely restored after 25 days. At the same time, the resynthesis of carbohydrates was reflected in a corresponding increase in the *lipid-free* dry weight. These results were confirmed and extended in another series of experiments in which the changes in lipids, alkali-stable carbohydrate, glycogen, and trehalose were examined at more frequent intervals between 0 and 45 days. The maximum range in values between the three to five experiments represented at each point in Fig. 1 is depicted by the vertical lines. Lipids continued to decrease slowly after the 25th day, as already reported (12). Carbohydrates, on the contrary, after resynthesis, were not used by the surviving infective embryo

TABLE IV

DRY WEIGHT, LIPIDS, AND CARBOHYDRATES IN UNEMBRYONATED, 10-DAY, AND 25-DAY EGGS*

	Days		
	0	10	25
	g. per 100 g. decoated, unembryonated egg solids		
Dry weight	100.0	89.0	83.5
Lipids	36.0	32.5	21.5
Carbohydrates†	15.4	8.7	15.4
Lipid-free dry weight‡	64.0	57.0	62.0

*Averages obtained from at least three experiments.
†Alkali stable, calculated as glucose.
‡By difference.

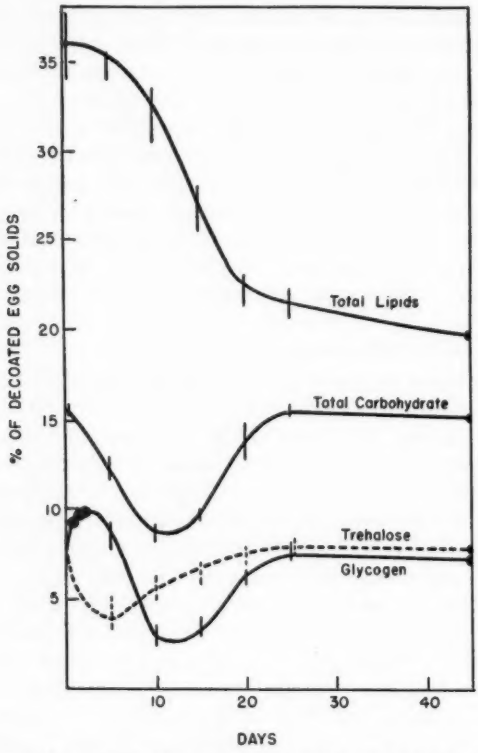

FIG. 1. Changes in total lipid, total alkali-stable carbohydrate, glycogen, and trehalose during embryonation of ascaris eggs at 30° C. The vertical lines represent the range in values obtained in a series of three to five experiments; the solid circles represent the results of a single experiment.

over periods extending well beyond the 45th day. Of the two component carbohydrates, glycogen increased somewhat for 2.5 days, decreased to a minimum at about 40% of its initial concentration in 10 days, and then increased in a regular manner. The trehalose concentration was at a minimum within 5 days, after which it, too, reappeared slowly.

If the protein nitrogen in the eggs (Table I) was converted to terms of protein, and to this was added the carbohydrates and lipids, these three classes of substances would account for more than 90% of the egg solids in 0-, 10-, or 25-day eggs. Of the remaining solids, some at least consisted of inorganic substances and some of the phosphorus compounds (Table III) whose concentration did not change greatly during embryonation. A small amount (7.9 mM.) of ether-soluble metabolic acids, present at 10 days, decreased to 3.3 mM. in 25 days, the difference (4.6 mM.) being small compared with the 37 mM. of resynthesized carbohydrate. More important, lipid-free solids increased concurrently with carbohydrates, suggesting that

the 10% of solids other than lipids, proteins, and carbohydrates was not contributing significantly to carbohydrate carbon. It seemed certain, therefore, that since protein carbon was not available for this purpose, the 222 mM. of new carbohydrate carbon must be supplied by the lipids, or from environmental carbon dioxide.

Carbon Dioxide Fixation

The effect of carbon dioxide on development was investigated in an experiment in which control eggs were embryonated for 15 or 25 days and compared with other samples from the same large batch in which respiratory carbon dioxide was continuously trapped in alkali. Control eggs were incubated in cotton-stoppered flasks in the presence of 1% sodium carbonate. The others were suspended in 0.1 *N* sulphuric acid in rubber-stoppered flasks containing a supply of filter paper saturated with potassium hydroxide the concentration of which was never less than 0.5 *N*. These flasks were gassed occasionally with carbon-dioxide-free air. It was found that the withdrawal of carbon dioxide from the atmosphere had no effect upon the rate of embryonation, and, as shown in Table V, was also without effect upon the metabolism of lipids and carbohydrates. The changes which occurred also conformed closely to those shown in Fig. 1. Such results, however, did not exclude the possibility that a part of the respiratory carbon dioxide was fixed in the egg before it could escape to the environment (see the Discussion) and an investigation with labelled carbon dioxide was therefore undertaken.

Four 5 g. egg suspensions, previously embryonated for 16 days, were washed and made slightly alkaline. Each flask, after receiving 52 μc. of C^{14}-sodium carbonate in a final volume of 15 ml. was sealed with a rubber serum bottle cap and acidified by injection, after which it was incubated for 24 hours. The egg suspensions were then gassed exhaustively with solid carbon dioxide and nitrogen, neutralized, and washed. Trehalose (and other small molecules) was extracted with hot water, and glycogen was prepared in the usual way from a homogenate of the residue. Both the

TABLE V

THE EFFECT OF LOW pCO_2 ON LIPIDS AND CARBOHYDRATES OF EMBRYONATING EGGS

	Days				
	0	15		20	
		Control	low pCO_2	Control	low pCO_2
	g. per 100 g. of decoated, unembryonated egg solids				
Lipids	34.0	26.0	26.0	22.0	22.0
Carbohydrate*	17.2	11.9	11.6	16.2	15.7
Glycogen*	7.9	3.8	3.4	7.1	6.7
Trehalose†	9.3	8.1	8.2	9.1	9.0

*As glucose.
†By difference.

TABLE VI

CARBON DIOXIDE FIXATION INTO GLYCOGEN AND TREHALOSE DURING THE 17TH DAY OF DEVELOPMENT*

	Fermentation products			C^{14} distribution					
				Glucose Carbon No.					
	CO_2	Acetic†	Lactic	1	2	3	4	5–6	
Glucose source	% theoretical			% of total activity					% recovery
Glycogen	96	74	99	1.2	3.9	46	51	0.9	103
Trehalose	93	71	87	1.5	5.4	44	49	0.9	101

*Average from two fermentations.
†Obtained by oxidation of ethanol.

glycogen and trehalose fractions were then hydrolyzed for 4 hours in *N* sulphuric acid, and glucose was isolated and fermented by *L. mesenteroides*. Although the degree of purity of the glucose obtained from such complex mixtures was doubtful, the results of the fermentation indicated that the purity was high (Table VI).

The average fixation in these four egg suspensions was 9.6% of the administered carbon dioxide, with 61% of the fixed carbon appearing in the carbohydrates. The specific activity (μc. C^{14} per mg. glucose) was higher in glycogen (0.094) than in trehalose (0.039). This difference no doubt arose in part, at least, from the differences in the rate of formation of these carbohydrates during the 17th day of development (Fig. 1). The yields of carbon dioxide (carbon 1) and lactic acid (carbons 4–6) from the fermentation were nearly quantitative (Table VI). The lower yield recorded for acetic acid was due in large measure to losses entailed in the oxidation of ethanol (carbons 2 and 3) to acetic acid, and in subsequent purification and isolation of the latter. Nearly all of the radioactivity of the glucose derived from both glycogen and trehalose was centered in carbons 3 and 4. As this type of distribution is well known in systems in which exchange rather than net fixation of carbon occurs (see the Discussion), the results of this experiment, considered together with those of the preceding experiment (Table V), did not support the hypothesis that carbon dioxide was a major source of carbohydrate carbon in the egg.

Carbon Balance

If, as now seemed probable, lipid carbon were being incorporated into carbohydrates without first being fully oxidized to carbon dioxide, then under controlled conditions it should be possible to demonstrate a balance between lipid carbon disappearing, on the one hand, and carbon dioxide and carbohydrate carbon appearing, on the other. To this end 30 g. of eggs were decoated and resuspended in 150 ml. of 0.5 *N* sodium hydroxide containing 6 ml. of sodium hypochlorite solution (6% available chlorine). After settling,

eggs and supernate were separated by decantation, and the eggs washed with sterile water. All subsequent operations were carried out aseptically. The egg suspension (50 ml.) was stirred magnetically while 10-ml. portions (6 g.) were removed into each of four 125 ml. flasks containing 20 ml. of 0.13 *N* sulphuric acid. After they were embryonated for 10 days, two of the egg suspensions were set aside at 3° C. The other two were gassed with air, and each of the flasks containing the suspensions was then connected through rubber stoppers and a glass U-tube to a second flask containing 10 ml. of 0.5 *N* sodium hydroxide. Each of the two flask pairs was sealed securely and connected with a water manometer and an oxygen supply. They were then returned to the incubator and embryonated for another 14 days. The slight negative pressure developing daily in each system owing to oxygen consumption by the eggs was relieved by admission of oxygen. At the conclusion of the experiment the two receiving flasks were removed and their carbon dioxide content determined. The egg suspensions were neutralized, washed, killed by heating, and homogenized. Dry weights, lipids, and carbohydrates were then determined. Carbohydrate carbon was calculated from the results of the anthrone analysis, lipid carbon by wet combustion. The results obtained from each pair of samples at 10 or 24 days, respectively, were all in good agreement. Volatile acids and glycerol were isolated after combining all the 10- and 24-day lipids, respectively.

The results of the dry weight, lipid, and carbohydrate determinations are included in Table VII primarily in order to show that in this experiment, as previously (Table IV), lipids decreased greatly between 10 and 24 days, whereas carbohydrates and lipid-free dry weights increased. More important was the near-perfect balance between the loss of lipid carbon (36.6 mg.) and the gain of carbohydrate and carbon dioxide carbon (37.6 mg.). Since only 72% of the disappearing lipid carbon appeared as carbon dioxide, with the remainder accountable as carbohydrate, and since carbon dioxide fixation did not appear to be important, it was concluded that a part of the disappearing lipids was converted to carbohydrates while still in a partially oxidized form.

TABLE VII

THE BALANCE BETWEEN CARBOHYDRATE AND LIPID CARBON IN 10-DAY EGGS, AND CARBOHYDRATE, LIPID, AND CARBON DIOXIDE CARBON IN 24-DAY EGGS*

	Dry weight, mg.			Carbon			Volatile acids		Glycerol
	Lipid-free residue	Carbohydrates†	Lipids	CO_2, mg.	Carbohydrates, mg.	Lipids, mg.	mM.	Carbon, mg.‡	Carbon, mg.
10	245	44.8	145	—	18.0	104.0	0.092	3.9	2.4
24	279	73.2	92	26.4	29.2	67.4	0.053	2.2	0.9
Change	+ 34	+28.4	−53	+26.4	+11.2	−36.6	−0.039	−1.7	−1.5

*Partial loss of the chitinous shell during treatment with hypochlorite required that the results be stated as mg./6 g. uterine eggs, rather than as g./100 g. decoated, unembryonated egg solids.
†Expressed as glucose.
‡Calculated from values in 14-day eggs (12) in which 1 mM. of mixed volatile acids was equivalent to 0.081 g., or 0.042 g. of carbon.

Discussion

Although the observation that protein and non-protein nitrogen remained essentially constant during embryonation of the ascaris egg was of considerable interest in itself, its main function in the present investigation was to direct attention to the need for defining the source of the carbon required in the synthesis of glycogen and trehalose in the period 10–24 days. The possibility that protein nitrogen remained constant, while protein carbon decreased, was rejected when it was found, by chromatographic methods, that the protein amino acids did not undergo noticeable qualitative changes during embryonation. Similarly, investigation of the non-protein nitrogen fraction did not support the possibility that these substances accumulated in insoluble form in the fraction analyzed for protein nitrogen, thus masking real changes in the protein. Conversion of the protein nitrogen in the protoplasm of the unembryonated egg (3.1 g.) to terms of protein (19.4 g.) and acceptance of the assumption that the usual fraction of this protein is glucogenic (35) would mean that about two-thirds of the protoplasmic protein would be required in order to provide sufficient glucogenic carbon for carbohydrate resynthesis. However, such a conversion could not occur without liberation of very large amounts of non-protein nitrogen, which, in fact, did not increase at all. Clearly, therefore, the protein and non-protein nitrogen fractions did not contribute significant amounts of carbon to the newly-formed carbohydrates.

Carbon compounds other than proteins, lipids, and carbohydrates probably comprised much less than 10% of the total egg solids, and did not decrease appreciably during development of the egg. Furthermore, the lipid-free dry weight increased, making participation of such compounds in carbohydrate renewal virtually impossible. The increase in lipid-free dry weight between 10 and 24 days was in reality direct evidence for the conversion of lipid carbon into carbohydrates, provided that respiratory carbon dioxide or carbon dioxide supplied by the ambient air was not extensively fixed by the egg.

The fact that eggs embryonated at very low partial pressures of carbon dioxide behaved identically with control eggs (Table V) could scarcely be accepted as conclusive evidence for the absence of fixation, as in an experiment of this kind partial pressures within the egg could not be measured or controlled. It was known, however, that a great deal of respiratory carbon dioxide did escape from the egg under identical conditions (Table VII). Furthermore, Passey (31) showed that the average respiratory quotient during the period in question was 0.69, a figure which agreed well with the R.Q. calculated for a typical triglyceride, rather than with a type of metabolism in which respiratory carbon dioxide was being fixed. These suggestions that net fixation did not occur were strongly supported when it was found that the carbon of C^{14}-carbon dioxide entered the glucose molecule primarily in the 3 and 4 positions. It is generally agreed that this distribution results from the fixation of carbon in the Wood-Werkman or similar reaction (30, 42) which is capable only of promoting a net exchange of carbon, rather than a net fixation.

By a process of elimination, therefore, the lipids remained as the only source of the carbohydrate carbon. This probability was further strengthened by the balance experiment in which the production of carbon dioxide and carbohydrate carbon equalled the loss of lipid carbon (Table VII). It was known from previous work (12) that only the triglyceride fraction was seriously involved in the catabolism of lipids of the embryo. Non-volatile and volatile acids had been isolated from this fraction, but it was not known with certainty whether the esterifying alcohol was glycerol. Schulz and Becker (36) found that the total lipids of the adult worms contained 8.8% glycerol, and since the greater part of these lipids occur in the reproductive system (11), the presence of glycerol in the egg was a reasonable inference which was confirmed in the present investigation. It is proper, therefore, to refer to the esterified acids which are metabolized by the embryo as triglyceride acids.

The final evidence linking triglycerides with carbohydrate synthesis was based upon the relation between oxygen consumption and triglyceride disappearance. Passey and Fairbairn (32) presented data for rates of oxygen consumption over an extended developmental period which were based upon egg samples prepared and handled identically with those employed in the present investigation. Integration of the rate curve between the 10th and the 25th day revealed that 0.81 mM. of oxygen was consumed per 100 g. of egg solids. This amount of oxygen could account for the complete oxidation of only 90% of the triglycerides known to disappear, without making any provision for other cell oxidations. Clearly, then, part of the triglycerides must be incompletely oxidized. It is equally clear (Table VII) that the greater part of this burden must be borne by the non-volatile acids. If completely converted to carbohydrate, glycerol carbon would provide 13% of the total amount required. On a similar basis, volatile acids would provide another 15%. It can be shown, however, from previously published results (12) that *glucogenic* carbon (3-carbon units) in these acids would provide only 4%, the remaining carbon being analogous in its behavior to that of the non-volatile acids.

Balance experiments performed in various ways on higher animals, or their tissues or organs, have repeatedly led to the conclusion that there is no net conversion of fat to carbohydrate in animal tissues (35). Lifson and associates (24) pointed out that if fatty acids were degraded to active acetate, condensed with oxaloacetate, and passed through the Krebs cycle, there could be no net carbohydrate synthesis, since 2 moles of carbon dioxide would be liberated for each mole of acetate condensed. Various investigators, however, have claimed that perfused cat liver (3), the fasting rat (6, 8, 24), and the lactating cow (22) can produce carbohydrate from butyrate, presumably following degradation of the latter to acetyl coenzyme A. If, as is probable, the long chain fatty acids in the ascaris egg are also oxidized with formation of acetyl-CoA, the same mechanism for conversion to carbohydrate may occur. Although such a mechanism is not yet established, recent evidence suggests that the reaction first proposed by Thunberg (40), in which

2 moles of acetate condense to form succinate, may be an important one. Indirect evidence for the presence of this mechanism in a number of organisms (19, 20, 41) was supported directly by Seaman and Nashke (37), who demonstrated the reversible cleavage of succinate to acetyl-CoA in *Tetrahymena pyriformis* and the presence of the enzyme in several tissues of the rat. This mechanism, or a similar one, would provide an efficient route for the incorporation of fatty acid carbon into carbohydrate, since as many as three of the four condensed carbon atoms could conceivably be available for the synthesis.

No intimate details of either fat or carbohydrate metabolism in ascaris eggs are available. The conversion of fats to carbohydrates appears to occur in preparation for some future requirement or demand of the larvae, for over long periods the surviving infective egg makes no use of the trehalose and glycogen which it has synthesized.

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FACTORS INFLUENCING THE AMOUNT OF INSULIN EXTRACTABLE FROM BEEF PANCREAS

I. EFFECTS OF AGING FRESH PANCREAS AT ROOM TEMPERATURE[1]

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Abstract

Effects on the concentration of extractable insulin of aging fresh beef pancreas at temperatures in the 22–26° C. range have been described. Two phases of change with time of aging have been observed, describable as a transient increase superimposed on a progressive fall toward zero in the concentration of insulin extractable from the pancreas. In two of the eight experiments reported only the second phase was detected.

Various hypotheses concerning the cause of the first phase are considered. The available experimental evidence is considered to support the hypothesis that the early rise in extractable insulin resulted from the continuing new formation of insulin from structural components already present in the cytoplasm of the beta cells at time of exsanguination, or diffusing into it thereafter. The progressive fall in the extractable insulin during the second phase must have resulted from destruction or inactivation of insulin within the pancreas.

The bearing of these recent findings on the usage of the term "The insulin content of the pancreas" has been discussed.

Introduction

Since the discovery of insulin in 1921 (2), the biochemical problems involved in its extraction and purification have occupied a prominent position in research, owing largely to the importance of purified preparations of insulin in the treatment of diabetes mellitus, in shock therapy, and in research. While many nutritional and endocrine factors affecting the amount of insulin extractable from fresh animal pancreas have been systematically studied and reviewed (4, 8), relatively little information has been published on the effects of various pre-extraction treatments of the pancreas following its excision from living subjects, or from man or animals at the time of death (6, 10, 12, 16, 17).

The present study on the effects of aging pancreas for varying periods of time after excision started as a control experiment during the collection of data on the insulin extractable from human pancreas (16). It was observed that, in fresh beef pancreas, a more or less regular sequence of changes occurred. The extractable insulin rose, in most cases, to a maximal value after 10–20 hours of aging at or near room temperature, and subsequently fell progressively toward zero in all cases. These phenomena of aging would not have been observed in the usual type of experiment in which the effects of various nutritional or endocrine factors on the insulin stored in the pancreas have been investigated, since, in these, any delay between excision and extraction has been studiously avoided.

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Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada.

Several variants of a basic pattern of experiment were used in the present study to provide evidence that the observed effects of aging were not artifacts. All such experiments will be described in order to avoid the difficulties inherent in basing conclusions on selected studies.

Materials and Methods

The following is a description of the basic pattern of the experiments to be reported. Healthy beef pancreas was obtained within a few minutes after the death of the animal by exsanguination. At that time the excised pancreas was trimmed as free as possible of adherent fat and lymph nodes and was subdivided with scissors into a number of samples, each weighing about 20 g. After they were weighed, the samples were deposited in individual numbered containers and were aged for different periods at a selected temperature before being extracted for insulin.

In some experiments samples were taken for histological study, and these were aged in individual containers to which Bouin's fluid was added at the same time as corresponding samples were extracted for insulin. At the end of 24 hours' fixation the blocks of tissue were dehydrated in isopropyl alcohol and embedded in paraffin in the usual manner. Sections of pancreas 5μ thick were stained by Gomori's chrome alum haematoxylin–phloxine stain and by Wilson's modification of Gomori's aldehyde–fuchsin stain (10). The latter method colors the beta-cell granules an intense purple, whereas the former stains the granules in the alpha cells red and those in the beta, blue. Sections were examined without the observer (W.S.H.) knowing any of the experimental details, and studies of beta-cell granulation were made according to methods previously published (19, 10).

TABLE I

SPECIAL FEATURES OF THE INDIVIDUAL EXPERIMENTS PERFORMED

Expt. No.	Description of animal	Method of obtaining samples of pancreas	Temp. at which aged (° C.)	No. of samples per point	Sterile technique used ?	Histology studied ?	Samples extracted at same time ?	Fig. No. where results shown
1	4-year-old Hereford steer	Single strips	25	1	No	No	No	1
2*a*	4-year-old Hereford steer	Pairs of strips	25	1	No	Yes	No	1
2*b*	4-year-old Hereford steer	Pairs of strips	25	1	No	Yes	No	1
3	7-year-old Ab-Angus cow	Diced and mixed	23.5	Initial 2; bal. 1	Yes	Yes	No	2
4	Ayrshire cow	Diced and mixed	22	2	Yes	No	Yes	3
5	2-year-old Shorthorn steer	Diced and mixed	23	2	Yes	No	Yes	3
6	1–5-year-old mongrel steer	Diced and mixed	23	2	Yes	No	Yes	2
7	2-year-old Hereford steer	Diced and mixed	26	2	Yes	No	Yes	3

In the transport of samples from the abattoir to the laboratory, use was made of a portable incubator which was thermally insulated and contained a large flask of water having the selected aging temperature. On arrival at the laboratory, the jars of aging pancreas were transferred from the carrier into a thermostatically-controlled incubator maintained at the same temperature.

In experiments 2*a* and 2*b*, separate series of insulin extractions were performed simultaneously by two technicians. In starting the extractions, one technician used the method of Scott and Fisher (14) in which the samples of pancreas were minced in hydrochloric acid–alcohol extraction fluid using sharp scissors (experiment 2*a*) while the other technician reduced the pancreas to very small fragments in extraction fluid by Waring blendor (experiment 2*b*). Separation of the extraction fluid from the pancreatic pulp was accomplished by pressing out the liquid through a filter of gauze in the first method, and by centrifugation in the second. The procedures correspond in all other respects. The precipitates of crude insulin were dissolved in equal volumes of 0.14 *M* sodium chloride adjusted to pH 2.5. Information concerning special procedures which were followed is given in Table I for each experiment.

In all experiments, estimations of the concentration of insulin in the acid saline solutions of crude extracted insulin were made to within an average standard error range of $\pm 10\%$. A mouse-convulsion method for the assay of insulin (16) which meets all of the requirements listed by Bliss (5) was employed.

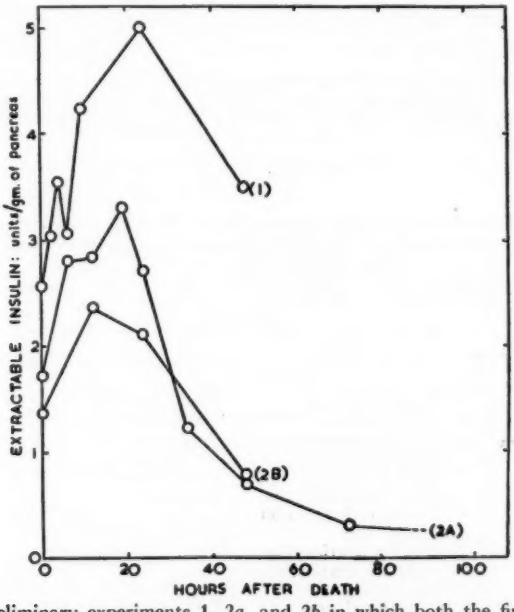

FIG. 1. Preliminary experiments 1, 2*a*, and 2*b* in which both the first and second phases of change in the extractable insulin of the pancreas with time of aging at room temperature were observed.

Results

The patterns of change with time of aging in the insulin extractable from pancreas in experiments 1, 2*a*, and 2*b* are illustrated in Fig. 1. Condensed descriptions of the histological conditions of the beta cells for the samples of experiments 2*a* and 2*b* are shown in Table II. Graphed results for other experiments are shown in Figs. 2 and 3.

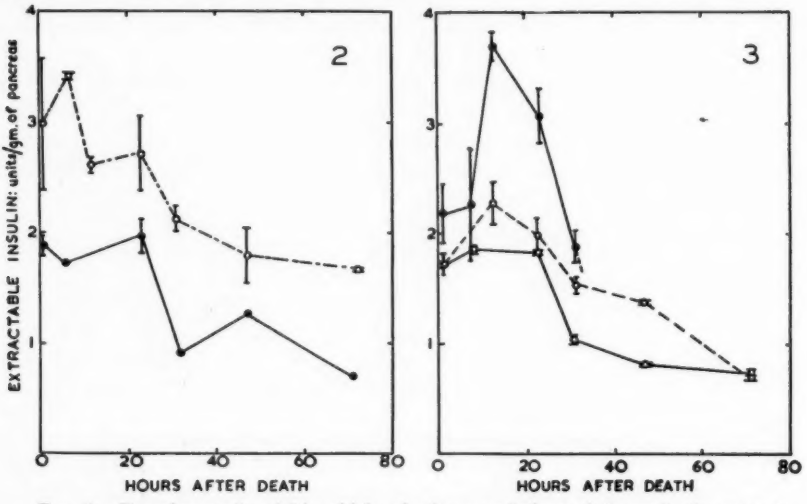

FIG. 2. Experiments 3 and 6 in which only the second phase of change in the extractable insulin of the pancreas with time of aging at room temperature was observed.

FIG. 3. Experiments 4, 5, and 7 in which both the first and second phases of change in the extractable insulin of the pancreas with time of aging at room temperature were observed.

TABLE II

OBSERVED CHANGES WITH TIME OF AGING AT 25° C. IN THE HISTOLOGICAL CONDITION OF THE BETA CELLS IN EXPERIMENTS 2*a* AND 2*b*, AND IN THE INSULIN EXTRACTABLE FROM THE PANCREAS

Aging time (hours from exsanguination)	Beta cells		Extractable insulin (units/g. pancreas)
	Cells	Stainable granules	
0.5	Well preserved	Well preserved	1.71
6.0	Well preserved	Well preserved	2.80
12.0	Well preserved	Well preserved	2.94
18.0	Well preserved	Well preserved	3.30
24.0	Well preserved	Well preserved	2.70
34.0	Cell boundaries disintegrating, nuclei pycnotic	Preserved	1.23
48.0	Cell boundaries gone, nuclei markedly pycnotic	Partially preserved	0.70
72.0	Only debris left	Islet granules seen in debris	0.32

Discussion

On the basis of the eight experiments performed, the aging of fresh beef pancreas in the 22–26° C. temperature range results in a transient early increase (phase I) superimposed on a progressive decrease in the concentration of extractable insulin within the pancreas (phase II). These two phases have also been observed in other experiments (7, 11, 18). The magnitude of the early increase ranged from negligibly small up to approximate equality with the amount of insulin initially extractable per gram of pancreas.

These findings might be interpreted in terms of several hypotheses, three of which will be considered. Before doing so it is noted that, in most of the experiments reported here and elsewhere (11, 18), the fresh pancreatic tissue was diced and the pieces were randomized before samples of them were taken for aging. Thus phases I and II were not caused by regional differences in the concentration of extractable insulin in the pancreas, such as are known to exist in the dog (3) and in man (17).

The apparent changes in the concentration of extractable insulin in aging pancreas could have resulted from progressive changes in the conjugation of protein or other pancreatic constituents with insulin. Conjugation of insulin with proteins can have marked effects on the intensity and duration of the hypoglycemic action of the former as in protamine-insulin or globulin-insulin, or in the binding phenomenon of Stadie (15). Since all insulin concentrations were determined by comparing the proportions of mice convulsing within 75 minutes after injection with either standard or test solutions of insulin, it is clear that such changes in the conjugation of insulin during aging might be reflected in assay results as apparent changes in insulin concentration.

Experimental evidence bearing on this hypothesis has been considered separately (11). The hypothesis has been found to be inadequate as a basis for explaining the observed changes with aging time in the amount of insulin extractable from beef pancreas.

The insulin extractable by the hydrochloric acid – ethanol medium employed might be considered to represent only part of a larger depot of insulin present in the pancreas at the time of its removal from the circulation. Suppose that, during aging, unextractable insulin having a concentration of I units per gram of pancreas becomes transformed at a rate K_1I into an extractable form, present in concentration C, and that the rate of inactivation of the extractable insulin is K_2C. (In other words the law of mass action is assumed to hold in both cases.) On the basis of these premises, and assuming that unextractable insulin is inactivated only after transformation into the extractable form, then the net rate of accumulation of extractable insulin in the pancreas is given by

$$dC/dt = K_1I - K_2C. \tag{1}$$

Integrating, the amounts of extractable insulin per gram of pancreas at any time, t, following its removal from the circulation would be given by

$$C = \frac{K_1I_0}{K_2 - K_1} \{ e^{-K_1t} - e^{-K_2t} \} + C_0e^{-K_2t}. \tag{2}$$

C_0 and I_0 represent the amounts of extractable and unextractable insulin per gram of pancreas at $t=0$, respectively, while K_1 and K_2 are positive velocity constants. Under these circumstances a pattern of change in the amount of insulin extractable per gram of pancreas which corresponds in both phases with the observed pattern and its variants would result.

An alternative hypothesis leading to the same mathematical formulation as is given in equation (2) can be set up. At the time of excision of fresh pancreas, the beta cells would contain certain amounts of substances in process of being synthesized into insulin as well as a certain amount of what might be described as stored insulin. In such living cells the synthesis of insulin might be expected to continue until some essential component of insulin-precursor materials or of available energy failed, or until the insulin-synthesizing mechanism within the cells ceased to function for other reasons. In the absence of the circulating blood the insulin synthesized in this way would accumulate and later would be inactivated within the pancreas.

In terms of this hypothesis, the rate of appearance of newly synthesized insulin in those beta cells in which the synthesizing mechanism remains intact might be assumed to be proportional to the concentration in the beta cells of that insulin precursor substance which is in shortest supply, and which thus forms the most effective limiting factor in the synthesis of new insulin. This assumption would require a different interpretation of factors K_1 and I_0 of equation (2) than was given on the basis of the preceding hypothesis, but no difference in the mathematical form of equation (2). In the present case I_0 would represent the concentration (expressed in terms of insulin molecules) of the limiting insulin-precursor substance(s) present in the beta cells of the pancreas at $t=0$, while K_1 would represent the velocity constant in the rate of production of insulin in the beta cells.

The magnitude of phase I in the change in concentration of extractable insulin with time of aging is represented by the first two terms on the right hand side of equation (2), and that of phase II by the last term. The assumptions used in setting up the algebraic formulations of the two preceding hypotheses are admittedly oversimplified. It is recognized that the observed changes in the concentration of extractable insulin are occurring in an extremely complex heterogeneous medium.

The relative strengths and weaknesses of the two hypotheses just stated can be considered from several points of view. First, in an experiment reported elsewhere (18), samples of the same pancreas were aged at 25° C. either fresh or after a short period at −70° C. The characteristic early rise in extractable insulin with time of aging was observed in the unfrozen samples but not in the frozen and thawed ones. On the basis of the hypothesis in which unextractable insulin is assumed to exist, this would entail the premise that the initial freezing and thawing of the fresh pancreas had quantitatively destroyed all of the unextractable insulin but none of the extractable insulin.

If, on the other hand, phase I is produced by the new formation of insulin, its extinction would be expected to follow the disruptive effects on beta cell structure of freezing and thawing. The latter explanation appears to be the more realistic of the two. The almost complete inhibition of incorporation of leucine-1-C^{14} into the insulin of incubating foetal calf pancreas by anaerobiosis, by 2,4-dinitrophenol, or by incubation for zero time, has led Light and Simpson (13) to believe that metabolic reactions were involved rather than a simple adsorption process. The part played by oxygen in the accumulation of extractable insulin observed in our experiments is under study.

Regardless of which, if any, of the above hypotheses has basis in fact, it is clear that the effect of aging on insulin extractable from beef pancreas requires some reinterpretation of the commonly used term "The insulin content of the pancreas". If transformation of unextractable into extractable insulin is the principal basis for the observed effects of aging, the insulin content of mature beef pancreas would be found to be severalfold larger than the insulin extractable from the pancreas at the time of slaughter. When the values $K_1=0.035$ per hour and $K_2=0.060$ per hour were fitted by inspection to the velocity constants of equation (2), and all values for the concentration of extractable insulin were normalized so that $C_0=1$, in each experiment, approximate agreement was found to exist between the time trend predicted by equation (2) and the experimental data of Fig. 1. If equation (2) fitted with these constants is interpreted in terms of a reserve of unextractable insulin, the insulin content of the pancreas (that is the extractable *plus* the unextractable insulin) would amount to more than six times the value for the extractable insulin at $t=0$ (i.e. $(I_0 + C_0)/C_0 = 6.1$). If, on the other hand, the increase in extractable insulin observed during phase I of the aging process is caused by the new formation of insulin, then the insulin content of the pancreas at $t=0$ would equal the insulin extractable from the pancreas, due allowance being made for processing losses during extraction.

On the basis of earlier studies which have been reviewed (8) it has become widely accepted that the stainable granules contained in the cytoplasm of the beta cells contain stored insulin. The granular material in the beta cells which is made visible by Gomori's stain shows a statistically significant one-to-one correlation with the extractable insulin of dog and of human pancreas (19, 10). If the acid alcohol method of insulin extraction removes only one-sixth, say, of the total insulin contained in the beta cells of fresh beef pancreas, can the remaining five-sixths of the insulin in the islets of beef pancreas (predicted by the one hypothesis but not by the other) be detected by histological means after extraction with acid alcohol?

To check on this possibility, some of the fragments of fresh beef pancreas from experiment 2 were examined for beta-cell granules or their debris following extraction with hydrochloric acid – ethanol. No such granules could be demonstrated using either Gomori's stain or phase microscopy on fixed tissue, although granules could be visualized by either technique using unextracted fixed tissue (9).

While the considerations presented above are suggestive rather than conclusive, it is our opinion that they favor a continuing activity of the insulin-synthesizing system in excised pancreas rather than a store of unextractable insulin which becomes extractable during aging. This view is supported by the finding of Grodsky and Tarver (7), who found that a net increase occurred in the amount of insulin extractable from sliced 1-g. samples of foetal beef pancreas incubated in a bicarbonate-buffered medium when oxygen was present, but not when it was replaced by nitrogen.*

If continuing new formation of insulin occurs when fresh pancreas is aged, then the time and extent of the rise in the extractable insulin of fresh beef pancreas during aging should be governed by three factors. These factors are: (i) the survival time of a functional insulin-synthesizing system within the beta cells; (ii) the concentration of some structural component or components of insulin already present in or otherwise available to the insulin-forming system at the time of exsanguination of the animals; (iii) the activity of insulin-destroying systems in the medium containing the insulin in the pancreas.

The microscopal studies showed that the boundaries, resolution of granules, and tinctorial differentiation of the islet cells were remarkably well preserved despite a post-mortem interval of 24 hours (but not of 34 hours) at 25° C. The *maximum* level of the extractable insulin occurred at about 18 hours after the excision of the pancreas (experiment 2). This cytological evidence is insufficient to indicate any specific changes in the beta cells which might be associated with a decreasing rate of insulin synthesis. However, it is clear that the production of the additional extractable insulin following excision of the pancreas occurred before disintegration of the cellular elements could be demonstrated by the staining methods employed.

The possibility that bacterial action may have caused or may have influenced the changes in the extractable insulin of the pancreas has not been specifically investigated. However, both phases I and II of change in the extractable insulin of pancreas during aging were observed in experiments where precautions were taken to prevent or minimize bacterial action (11, 18) as well as in experiments where such precautions were not taken (Table I). This is taken as an indication that these features of the aging effect are not primarily due to bacterial action.

To summarize, the data reported in this paper support the view that the aging of untreated fresh beef pancreas is frequently accompanied by an early increase in the insulin extractable therefrom, or, in other cases, by a transient delay in the subsequent progressive fall. However, the inability to detect this first phase in two of the eight experiments raises special problems. These relate on the one hand to the reality of such a phenomenon and, if it is real, to what conditions affect the magnitude of the rise. The results of other experiments (7, 11, 18) strengthen the view that the phenomenon is a

*The time after death of the foetus when these phenomena were observed was not reported, but since comparison was made with the studies in perfused rat pancreas of Anderson and Long (1), it would appear that *fresh* foetal pancreas was employed.

real one, and that it results from the continuing synthesis and accumulation of insulin in the beta cells of the excised pancreas.

If this is true, then the meaning of "The insulin content of the pancreas", a term found in published studies which have been reviewed (8), requires clarification. In most of the published studies employing this term the pancreas was excised and frozen, or insulin extraction was started either at time of death of a freshly-killed animal or at time of excision of the pancreas from a live animal. We propose that the above term should refer only to this time, because of widely variable changes which occur in the extractable insulin of the pancreas during aging in the absence of its blood supply. The possibility that equality may exist between the insulin content of the pancreas and the insulin extractable therefrom by a given procedure is an attractive one, but requires separate confirmation.

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FACTORS INFLUENCING THE AMOUNT OF INSULIN EXTRACTABLE FROM BEEF PANCREAS

II. EFFECTS OF ALTERING THE EXTRACTION PROCEDURE ON THE CHANGES IN THE INSULIN EXTRACTABLE FROM AGING PANCREAS AND ON RECOVERY OF INSULIN[1]

C. J. LABUSCHAGNE,[2] B. K. HAESSIG, AND GERALD A. WRENSHALL

Abstract

Using variants of a hydrochloric acid – ethanol extraction procedure, which are described, only about one-tenth of the crude insulin extractable from beef pancreas was found to be extracted in the same way as crystallized Zinc-Insulin added to extraction fluid. An effective conjugation of most of the extractable crude insulin from beef pancreas with non-insulin factors thus appears to have occurred at least during the acid–alcohol phase of extraction. Nevertheless the mouse-convulsion method used for insulin assay proved effective in estimating the amount of this conjugated fraction of the extractable insulin following the addition either of the unconjugated Zinc-Insulin-Toronto or of the conjugated Protamine Zinc Insulin (Toronto) to the crude insulin in acid alcoholic extracts of pure beef pancreas. The phases of transient increase and progressive decrease in the insulin extractable from initially fresh beef pancreas during aging also occurred in the conjugated fraction of the extractable insulin. The proportion of this crude insulin which extracted as conjugated insulin did not change appreciably during the process of aging.

The above findings support the view that the transient increase superimposed on a progressive decrease in the extractable insulin during aging of fresh pancreas are not caused by progressive changes in conjugation of insulin. The finding of similar changes with time of aging in the concentration of extractable insulin of beef pancreas when two different extraction procedures were employed, and again when two different extraction media were employed, suggests that these changes are not products of the method or materials employed for extraction.

Introduction

Effects of aging fresh beef pancreas at room temperature on the insulin extractable therefrom have been described in a previous publication (4). These effects consisted, in most cases studied, in a transient increase having a maximum value at about 18 hours superimposed on a progressive fall toward zero as aging proceeded. Several hypotheses to explain these changes were proposed and their relative merits considered.

In one of these, it was proposed that changes in the combination of insulin with normal components or with decomposition products of pancreas might cause a progressive increase in the hypoglycemic effect of the insulin during the first phase of the aging effect. For example, it is possible that a given amount of crude insulin extracted from fresh pancreas might be so conjugated with non-insulin substances that its hypoglycemic activity would be retarded more than if it were extracted from a sample of the same pancreas aged for 18 hours at room temperature. Under such conditions a fixed amount of insulin extracted from pancreas after different aging periods might give

[1]Manuscript received January 14, 1957.
Contribution from The Banting and Best Department of Medical Research and the Department of Physiology, University of Toronto, Toronto, Canada.
[2]Nuffield Foundation Fellow.

different results in terms of the frequency of hypoglycemic convulsions in mice observed for only 75 minutes after injection.

It is the objective of the present paper to test the validity of the above hypothesis. For this purpose, both the aging phenomena and the recovery of purified insulin added to the extraction fluid or to alcoholic extracts of beef pancreas have been studied using the hydrochloric acid – ethanol method of Scott and Fisher (2), and modifications thereof to be described.

The results of aging observed using this method have also been compared with those obtained using a phosphoric acid – ethanol method for insulin extraction (1).

Materials and Methods

The general procedure followed in this paper has already been described (4). The standard or *A* extraction procedure followed in the present series of experiments was started in each case by freezing (to $-10°$ C.) each weighed sample of pancreas following its period of aging. Each frozen sample was cut into thin shavings, fragmented further using a chilled microtome knife, and the frozen fragments were added to the extraction fluid (4 ml. per g. of pancreas), all extractions proceeding concurrently. After 2 hours the fragments of pancreas were separated from the extraction fluid by straining the latter through a double layer of gauze. An equal volume of fresh extraction fluid was then added to the solid residue and a second extraction was carried out. After 2 hours, the second lot of extraction fluid was separated from the solid residue by means of a mechanical press. The two lots of extraction fluid were combined, and the total volume was measured. A 50 ml. volume of this solution of crude insulin was adjusted to pH 8 using concentrated ammonium hydroxide and was filtered through a Whatman No. 1 paper.

A 40.0 ml. aliquot of the filtrate was transferred to a large (250 ml.) centrifuge pot to which was added 70 ml. of absolute ethanol and 110 ml. of ether to precipitate the insulin. After they had been allowed to stand overnight at 3–5° C., the pot and contents were centrifuged for half an hour at 2000 r.p.m. The clear supernatant fluid was discarded. The precipitate was dissolved in 20.0 ml. of 0.14 *M* sodium chloride, the final pH being adjusted to 2.5 by the addition of a small amount of concentrated hydrochloric acid. The solution was assayed for insulin in this form using a mouse-convulsion method (3) after suitable dilution with 0.14 *M* sodium chloride at pH 2.5. The average standard error of concentrations of insulin, as determined by assay, was $\pm 10\%$.

Two other preparations of insulin were obtained from another aliquot of the alcoholic extract of crude insulin from which the *A* fraction was obtained. Approximately 50 ml. of the acid alcoholic solution were centrifuged for 30 minutes at 2000 r.p.m. to deposit undissolved matter. A 40.0 ml. volume of the clear supernatant solution was placed in a large centrifuge pot, and 70 ml. of absolute ethanol plus 110 ml. of ether were added as in the *A* extraction. After overnight refrigeration at 3–5° C., the pot was centrifuged

for 30 minutes and the supernatant fluid transferred into a second centrifuge pot. The remaining precipitate was dissolved in 20.0 ml. of 0.14 *M* sodium chloride and the pH adjusted to 2.5. The insulin of this solution was designated as the *B* fraction.

Concentrated ammonium hydroxide was next added dropwise to the clear acidic supernatant ethanol–ether extract described in the preceding paragraph until pH 8 was reached, as judged by pH paper (p Hydrion, Cenco). During the course of this addition a precipitate formed which was allowed to settle overnight at 3–5° C. It was then spun down for 30 minutes at 2000 r.p.m. and the clear supernatant fluid decanted. The precipitate was dissolved in 20.0 ml. of 0.14 *M* sodium chloride at pH 2.5, and was then ready for insulin assay. This was designated as the *C* fraction of the extractable insulin of the pancreas.

A study of the above procedures indicates that *B plus C* fractions of the extracted insulin might be expected to correspond in amount to the *A* fraction. The basic reason for introducing studies with the *B* and *C* extraction procedures described above is to illustrate that crystallized Insulin-Toronto by itself does not react in the same way as crude beef insulin or as Insulin-Toronto added to crude beef insulin in acid alcohol.

In experiment No. 1, Zinc-Insulin-Toronto (lot 855-7) was added in graded amounts (0, 4, 20, 80 units) to duplicate 40.0 ml. volumes of the fresh hydrochloric acid–ethanol extract of a beef pancreas that had been frozen prior to extraction. Forty units of Protamine Zinc Insulin (Toronto) were added to 90.0 ml. of the fresh extract from which two 40.0 ml. aliquots were then taken. One from each pair of these samples was then extracted immediately by the procedure described above to obtain the *A* extract. The other 40.0 ml. volume of each solution was processed immediately to yield *B* and *C* extracts.

Forty units of either Zinc-Insulin-Toronto or Protamine Zinc Insulin (Toronto) were added to 40.0 ml. samples of unused acid alcohol extraction fluid, and the mixtures were re-extracted to yield *B* and *C* insulin fractions (experiment No. 2).

In experiment 3, *A*, *B*, and *C* extractions were made on the hydrochloric acid – ethanol extracts from samples of initially fresh mature beef pancreas aged for different periods of time. The aging temperature was 26° C. In this experiment the pancreas was cut with sharp scissors into pieces weighing approximately 1 gram each. Pieces were randomized, and lots weighing approximately 25 grams each were deposited in Petri dishes, each of which contained a 10 ml. volume of sterile 0.14 *M* sodium chloride saturated with thymol.*

In experiment No. 4 the effects of aging samples of fresh pancreas at 22° C. on the concentration of extractable insulin were compared using two

*The hydrochloric acid – ethanol extraction fluid used in this experiment was prepared with a sufficiently increased concentration of ethanol to render the concentration of ethanol in the mixture of ethanol plus saline equal to that of the standard extraction fluid of Scott and Fisher (2).

different methods. The first of these was the standard hydrochloric acid – ethanol or *A* method of extraction described earlier. The second was a phosphoric acid – ethanol method (1). All operations with fresh pancreas were carried out over fresh waxed paper, using autoclaved rubber gloves and surgical instruments and using sterilized glass containers for samples of pancreas.

Results and Discussion

The recovery of Zinc-Insulin-Toronto following its addition to freshly extracted crude insulin in acid alcohol (experiment No. 1) is illustrated in Fig. 1. The straight lines shown in Fig. 1, relating the number of units of added Insulin-Toronto to the total number of units of insulin recovered, were fitted using the method of least squares.

It is noted that most of the added insulin was recovered. This is true for the *B plus C* extractions as well as for the regular *A* extractions. From 35 to 90 % of the extractable insulin appeared in the *B* fraction, the percentage increasing progressively with the concentration of added insulin. However, in every case the amount of insulin recovered in the *B plus C* extraction fell slightly below that of the corresponding *A* extraction. This apparently characteristic difference could have been caused either by the loss of slightly more insulin in the preparation of *B* and *C* samples than in the *A* samples, or by differences in the assayability of the different fractions by the mouse-convulsion method. In either case the difference is a small one, and appears insufficient either to cause or to modify greatly the observed effects of aging fresh pancreas on the amount of insulin extractable and assayable from it.

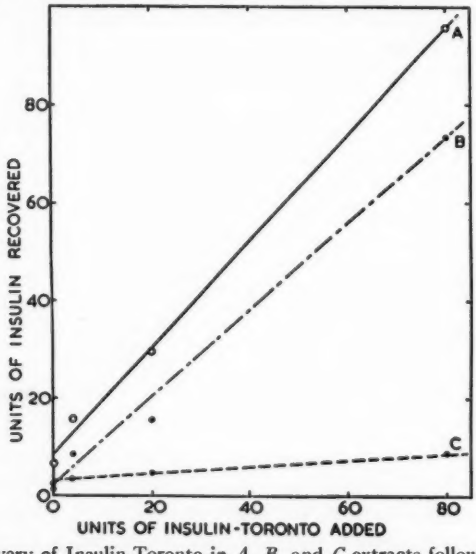

FIG. 1. Recovery of Insulin-Toronto in *A*, *B*, and *C* extracts following the addition of measured amounts to freshly extracted crude beef insulin in hydrochloric acid – ethanol extraction fluid.

When Zinc-Insulin-Toronto was added to unused hydrochloric acid – ethanol extraction fluid with *B* and *C* extractions subsequently performed (experiment No. 2), approximately three-quarters of the insulin was recovered in the *C* portion. None was recovered in the *B* extract as judged by the mouse-convulsion method of assay. When Protamine Zinc Insulin (Toronto) was added to the acid alcoholic extract of beef pancreas (experiment No. 1), 108% and 94% of the added insulin was recovered in the *A* and in the *B plus C* extracts, respectively. Of this recovered insulin, 76% was found in the *B* extract. When Protamine Zinc Insulin (Toronto) was added to unused extraction fluid (experiment No. 2) only 40% of the added insulin could be detected by assay. Of this recovered insulin, 81% was found to be in the *B* extract with the remaining 19% appearing in the *C* portion. These distributions between *B* and *C* fractions are similar to those observed when crude insulin was extracted from aging beef pancreas (Fig. 2).

Changes with time of aging in the concentration of insulin in the *A*, *B*, and *C* extracts from initially fresh beef pancreas (experiment 3) are illustrated in Fig. 2. The same sequence of changes in the insulin extractable from pancreas as was reported earlier (4) is again apparent in both the *A* and the *B plus C* plots. In Fig. 2 the predominant and trend-determining extract is clearly the *B* one.

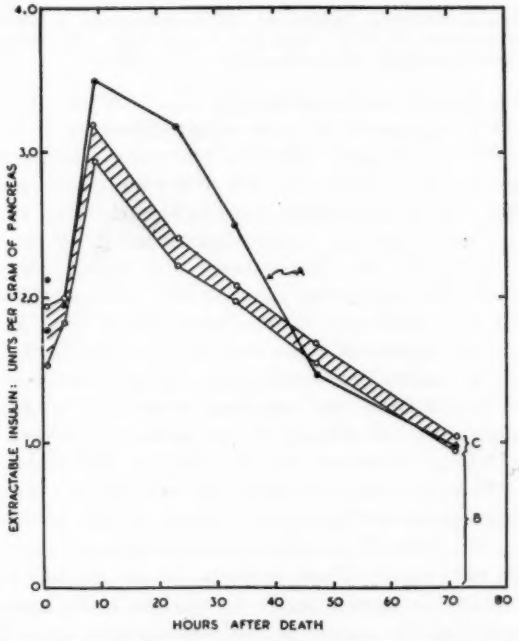

FIG. 2. Effects of aging beef pancreas at 26° C. on the concentrations of the extractable insulin in the *A*, *B*, and *C* extracts.

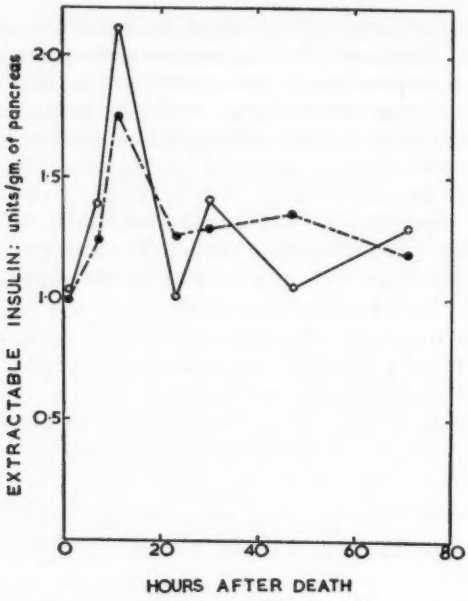

FIG. 3. Effects of extraction medium on the concentrations of insulin extractable from aging beef pancreas at 22° C. Full line: hydrochloric acid – ethanol extraction fluid. Broken line: Phosphoric acid – ethanol extraction fluid.

Aside from the general correspondance in Fig. 2 between the *A* and the *B plus C* trends, there appears to be a secondary difference in the interval between 10 and 30 hours of aging following removal of the pancreas from its circulating blood supply. However, this difference does not appear to be important relative to the hypotheses considered earlier (4), since it has not reappeared in the same form in another exper ment (5) of the same type as that illustrated in Fig. 2. No characteristic difference in the extractability of insulin from beef pancreas during aging at 22° C. was noted when different extraction media were employed in experiment No. 4 (Fig. 3).

To summarize, the results of experiment No. 1 indicate that none of the combinations of the added Zinc-Insulin-Toronto or Protamine Zinc Insulin (Toronto) with factors from beef pancreas present in the extraction fluid interfered seriously with the efficacy of the mouse-convulsion method used to measure the insulin. This was true not only for the regular *A* extraction but also for the *B* and *C* portions, extracted and assayed separately. The early rise and subsequent decline in the amount of insulin extractable were observed in both the *A* and *B* extracts, and were also seen when two different extraction media were used. These findings do not support the hypothesis that the aging effects are caused simply by changes in the speed of action of insulin that result from its manner of combination with other factors in the extraction fluid. They do not, of course, completely eliminate this possibility.

Acknowledgments

The authors wish to thank the Swift Canadian Company Limited, in Toronto, for their friendly co-operation in providing access to the fresh beef pancreas used in this study, and Miss Isabell Jasper for technical help with insulin extractions and assays. In particular they are grateful to Professor C. H. Best for his encouragement and advice during the course of this project, and to Dr. G. R. Williams for suggestions concerning terminology.

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FACTORS INFLUENCING THE AMOUNT OF INSULIN EXTRACTABLE FROM BEEF PANCREAS

III. EFFECTS OF TEMPERATURE AND OF FREEZING AND THAWING ON CHANGES ACCOMPANYING THE AGING OF FRESH PANCREAS[1]

GERALD A. WRENSHALL, W. G. BRUCE CASSELMAN,[2] AND CHARLES H. BEST

Abstract

The early rise with time of aging in the amount of insulin extractable from fresh beef pancreas has been observed in two different extracts, both at 6° C. and at 25° C., in samples of the same mature beef pancreas. The same phenomenon has been seen to a lesser degree at 25° C. in samples of fresh pancreas from immature animals. In the study with pancreas from the mature animal the rates of change in extractable insulin were 12 times as large at 25° C. as at 6° C. The early rise was observed in fresh pancreas but not in samples which had been frozen and thawed prior to aging.

Introduction

The results of aging fresh beef pancreas under standardized conditions (2, 6) have focused our interest on the factors previously kept constant. Samples of the same pancreas have now been aged at two widely different temperatures, and the effects of freezing and then thawing other samples on their response to aging have been observed. The effects of aging on the insulin extractable from pancreases of mature and of growing animals have been compared. Experiments were performed at 6° C. and 25° C., and great care was taken to minimize bacterial contamination.

Materials and Methods

The pancreas of a 6-year-old cow was removed from the freshly opened abdomen, following exsanguination. The pancreas was handled with sterilized rubber gloves and was diced over fresh waxed paper, using sterilized scissors. The pieces were then randomized. Samples of pancreas having approximately equal mass were weighed out and were enclosed in sterile incubation jars, each containing 10 ml. of 0.14 *M* sodium chloride saturated with thymol. Some samples were frozen between slabs of solid carbon dioxide before being thawed and allowed to age at 25° C. The remaining samples of pancreas were divided into two groups, one group being maintained at 6° C. and the other at 25° C. until required for extraction of insulin.

The fresh pancreases of four 6-week-old bull calves were obtained and processed under similar circumstances, and samples were allowed to age at 25° C. Insulin extractions of types *A*, *B*, and *C* described earlier (2, 3) were made on all samples of pancreas aged at 25° C. Type *A* extractions were performed on samples aged at 6° C.

[1]Manuscript received January 14, 1957.
Contribution from The Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada.
[2]Senior Medical Research Fellow, National Research Council, Canada.

Results

The amount of insulin extractable from mature beef pancreas aging at 6° C. rose to a peak value at about 8–12 days and then decreased during the subsequent 18 days (Fig. 1). In other samples of the same pancreas aged at 25° C. there was a similar increase of about 50% in the amount of extractable insulin. This was reached at about 18 hours after death. The concentration

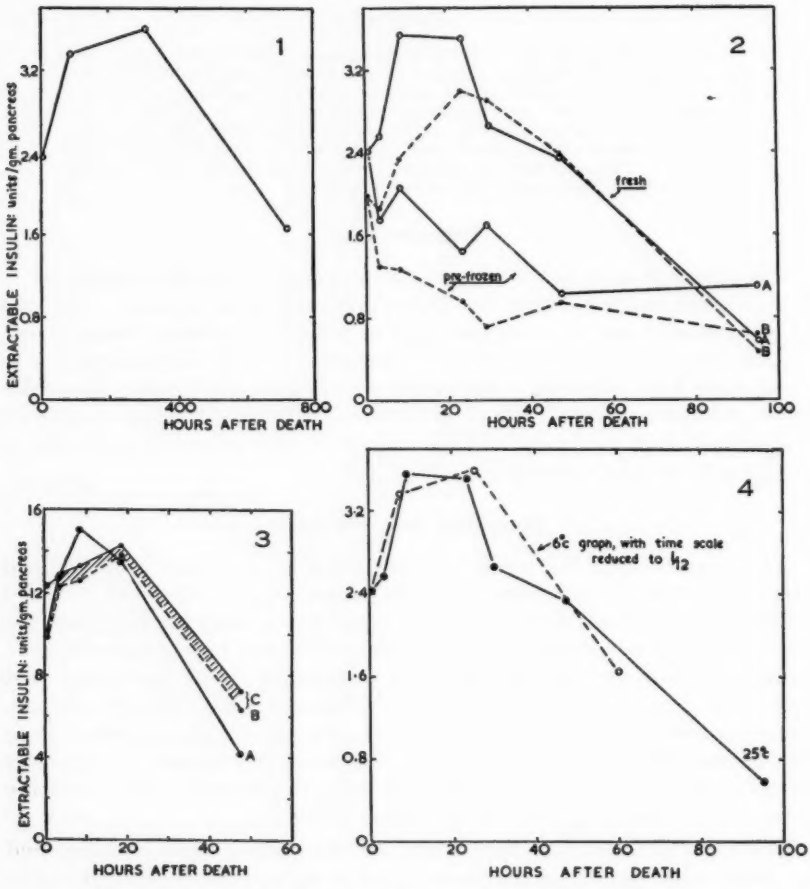

FIG. 1. Effects of aging samples of fresh cow pancreas at 6° C. on the concentration of insulin extractable.

FIG. 2. Effects of aging fresh and frozen and thawed samples of cow pancreas at 25° C. on the concentration of insulin extractable in *A* and *B* fractions therefrom. All samples of pancreas for Figs. 1 and 2 came from the same animal.

FIG. 3. Effects of aging at 25° C. on the insulin extractable from the pancreases of six-week-old bull calves using the *A*, *B*, and *C* procedures.

FIG. 4. Comparison of the effects of aging samples of fresh cow pancreas at 25° C. (full line) and at 6° C. (broken line) on the insulin extractable therefrom (*A* extracts). The time scale of the graph for 6° C. is condensed to 1/12th of its actual value.

then fell progressively toward zero. This was true of both the *A* and the *B* fractions (Fig. 2). In the samples of the same pancreas which were rapidly frozen within half an hour after the death of the animal, thawed 1 hour later, and aged under the same conditions as the fresh pancreas, no increase in extractable insulin was observed (Fig. 2). The concentration of insulin in all *C* type extracts were at or below the lower limit for assay by the mouse-convulsion method used.

In the pancreases from the 6-week-old calves, an increase in extractable insulin was observed at about 12 hours of aging at 25° C. (Fig. 3). The lack of prominence in this increase appears to have been caused by a high rate of disappearance of extractable insulin during aging. As was reported previously (2), the *B* fraction of the extractable insulin was approximately equal in amount of the *A* value in all cases studied.

Discussion

The new findings of greatest interest arising out of these experiments are: (*a*) the close similarity in the pattern and sequence of changes in the concentration of insulin extractable from mature beef pancreas aged at 6° C. and at 25° C.; (*b*) the 12-fold difference in the rates at which the sequence of changes takes place; (*c*) the suppression in both *A* and *B* extracts of the phase of early increase in extractable insulin during aging, caused by initial freezing and thawing of samples from the above pancreas.

Finding (*b*) demonstrates that the factors responsible for the early rise and coincident progressive fall, during aging, in the concentration of extractable insulin are temperature-sensitive. Finding (*a*) indicates that these factors have approximately the same temperature coefficients.

In order to obtain a figure for the average temperature coefficient, Q_{10}, for these aging effects in fresh pencreas, the rate of accumulation of extractable insulin in the pancreas at time of excision was estimated at 25° C. relative to that at 6° C. Designating these rates as R_{25} and R_6, respectively, inspection of Fig. 4 shows that $R_{25}/R_6 = 12$. From this figure $Q_{10} = (R_{25}/R_6)^{\frac{10}{25-6}} = 3.6$. This value is higher than the temperature coefficients for most biological processes, which have Q_{10} values between 2 and 3, and is much higher than is usually observed in physical processes (1).

The rate of accumulation of extractable insulin in the freshly excised beef pancreas at body temperature, as estimated by extrapolation of this figure to 37° C., corresponds in magnitude with the rate at which insulin is required by the unanaesthetized depancreatized dog (5). However, speculation on the significance of this correspondence would be out of place here owing to the large and unexplained range of variation in the extent and rate of the initial increase in extractable insulin of beef pancreas during aging at room temperature (6). The possibility that this variation might result from differences in the time interval during which the exsanguinated pancreas remains at 37° C. in the carcass assumes increased importance because of the above finding of temperature dependence, and is currently under investigation.

The well-established high initial concentration of insulin in calf pancreas relative to that in grown animals is apparent when Figs. 2 and 3 are compared. On the other hand the size of the transient net increase during aging at 25° C. in the concentration of the insulin extractable from calf pancreas is small relative to the initial value when compared with that seen in the average mature animal. However, for reasons considered in the preceding paragraph, it is pointless to speculate at present concerning the cause of this difference.

No satisfying explanation for the suppression of the phase of early increase in extractable insulin (seen in Fig. 2) during aging after freezing and thawing has been devised in terms of *unextractable* insulin. On the other hand the extinction of this phase, following disruption of cell boundaries by freezing and thawing (4), is what would be expected to occur if the early increase in extractable insulin resulted from the completion of synthesis of new insulin from precursor materials available to the insulin-synthesizing centers within the excised pancreas (6).

To summarize: The early increase and subsequent decrease in the insulin extractable from initially fresh beef pancreas occurred to similar extents but with rates differing 12-fold in magnitude when samples of the same pancreas were aged at 6° C. and at 25° C. Preliminary freezing and thawing of other samples eliminated the early increase and may have slowed the rate of decrease in extractable insulin. Both the transient early increase and the progressive decrease in the amount of insulin extractable from calf pancreas during aging at 25° C. have been observed, the latter phase being large relative to the former.

Acknowledgments

The authors wish to thank Dr. G. R. Williams for many interesting discussions of the findings. Technical support with insulin extractions and assays was provided by Miss Isabell Jasper and Miss Patricia Dolan.

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Symposium on the Ultrastructure of Cells

This symposium was held on October 20, 1956, at the 20th Annual Meeting of the Canadian Physiological Society in the Université de Montréal, Montreal, Quebec. Dr. Leonard F. Bélanger, Professor of Histology and Embryology at the University of Ottawa, acted as Chairman. The symposium was organized by a committee under the chairmanship of Dr. Eugène Robillard, Professor of Physiology at the Université de Montréal.

Readers of this symposium may be interested to know that other aspects of the subject were discussed at a symposium on "The Structure and Function of Subcellular Components" which was held by the Biochemical Society, Great Britain, in February 1957, and the papers will be published as a Report of the Biochemical Society.

THE FINE STRUCTURE OF CELLS[1]

A. F. Howatson and A. W. Ham

During the past few years there has been a remarkable increase in our knowledge of the cell especially in relation to the structure and function of its cytoplasmic components. The advances have been due in large measure to the development of two new cytological techniques (*a*) the technique of thin sectioning, which enables the high resolving power of the electron microscope to be applied to the study of the structure of intact cells, and (*b*) the technique for the separation of cell components by differential centrifugation, which has yielded important information about the chemical constitution and enzymatic activities of cell organelles. These two techniques are to some extent complementary, the findings of one being helpful in illuminating those of the other.

The primary concern of this paper is with the results obtained by the first method, but structure and function are closely interwoven, and the previously sharp boundaries between cell morphology on the one hand and cell physiology and biochemistry on the other are gradually disappearing.

Before describing what has been revealed by the electron microscope it may be helpful to recall the appearance of the various cell organelles and inclusions as seen in the light microscope. Several techniques of fixation and staining are required to demonstrate these bodies, not all of them being ordinarily visible in any given preparation. Fig. 1 is a kind of composite diagram of a cell in which the principal components revealed by the various methods are depicted. These include: in the cytoplasm, the cell membrane, chromidial substance, Golgi complex, centrioles, mitochondria, fibrils, secretory granules, and stored material such as glycogen or fat; in the nucleus, the nuclear membrane, nucleolus, and chromatin granules.

We propose to take up these cell components in turn, to describe their appearance as seen in electron micrographs, and to discuss briefly the implications of the findings.

[1]Manuscript received March 27, 1957.
Contribution from the Department of Anatomy, University of Toronto, Toronto, Canada. This paper was presented at the Symposium on the Ultrastructure of Cells held as part of the 20th Annual Meeting of the Canadian Physiological Society, Montreal, Quebec, October 20, 1956, and is based in part on a paper given at the Second Canadian Cancer Conference, Honey Harbour, June 1956 (Proceedings of the Second Canadian Cancer Conference (in press), Academic Press Inc., New York).

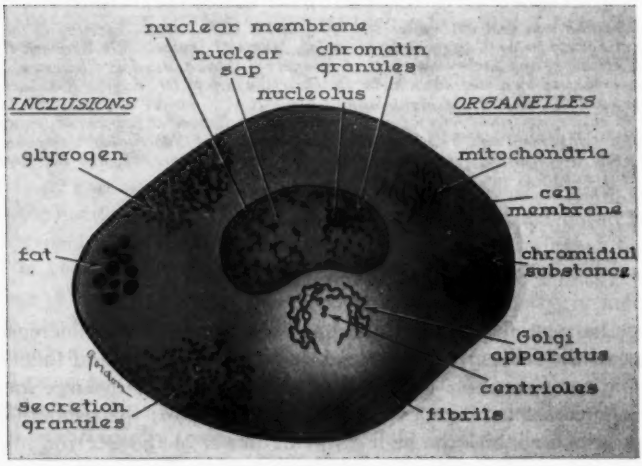

FIG. 1. Diagram of a cell showing components demonstrable by light microscope techniques.

Unfortunately we are immediately faced with difficulties of terminology. As new structures were discovered in cells, new terms were introduced to designate them. Not all investigators who were working in this field used the same terminology and to complicate matters still further many of the first-used terms were found, as knowledge advanced, to be less apt than when they were coined. As a consequence the terminology used in connection with some of the cytoplasmic organelles is in a state of flux. This is particularly so in the instance of the cytoplasmic membrane systems, which we consider first. Perhaps the best way to explain the terms employed is to review briefly how and why they came into use.

The Cytoplasm

In some cells there are regions of the cytoplasm that stain similarly to the chromatin of the nucleus. Because of this property the substance contained in these regions was called *chromidial* or *chromophile* substance. When acid and basic dyes were distinguished from one another, it was observed that both chromatin and chromidial substance combined with basic rather than with acid dyes, and this led to chromidial substance being called the *basophilic component* of the cytoplasm. In 1900 Garnier (8), noticing that the amount of chromidial substance differed in different types of cells, and that its presence in substantial quantities was associated with cells performing special kinds of work, gave it still another name, *ergastoplasm*. In more recent years this same material has been studied by ultraviolet absorption methods and shown to contain ribonucleic acid (RNA), and so it is sometimes referred to as *cytoplasmic RNA*.

Before the advent of electron microscopy little was known about the structure of the basophilic component, but during the past few years its structure has been revealed in increasing detail, and still more terms have been introduced to describe it. In order to understand the new terms it is necessary to comment briefly on the early electron microscope work.

In 1945, before thin sectioning procedures were developed, Porter *et al.* (24) examined thinly spread areas of whole cells grown *in vitro*, and noticed that a 'lace-like reticulum' with vesicles distributed along it could be seen in the cytoplasm. Subsequently Porter and Kallman (23) referred to this component as *endoplasmic reticulum*, the word 'endoplasmic' being chosen because in these very thinly spread cells the reticulum did not extend to the outer regions of the cell close to the cell membrane (the ectoplasm).

When thin-section methods became available a component of the cytoplasm was observed which was later identified with the endoplasmic reticulum that had already been observed in thinly spread cultured cells. This was described first as fibrils or filaments and later as lamellae. Dalton *et al.* (6), and independently Bernhard and others (4) observed that concentrations of the filaments or lamellae corresponded in location within the cell to the basophilic component. It became generally accepted at that time that the lamellae or endoplasmic reticulum seen in electron micrographs was the counterpart of the basophilic component seen with the light microscope.

As technical procedures improved, however, it became clear that the filaments seen in some cells that first appeared as single and then as double lines were in fact sections of very flattened membranous vesicles or sacs (Fig. 2). In other types of cell the vesicles may be distended to a greater or less extent (Fig. 3).

The term *endoplasmic reticulum*, however, was not universally adopted to describe these membranous structures. Some workers thought that a term applicable to both the chromidial substance of the light microscope and the so-called endoplasmic reticulum of the electron microscope would be preferable, and for this purpose *ergastoplasm* met most favor and began to come into fairly general use. Others again, preferred to use purely descriptive terms such as intracellular cytoplasmic membranes or cytomembranes to describe these structures.

As resolution in micrographs improved still further it became clear that the ergastoplasm had a granular component as well as a membranous one. These granules, first clearly described by Palade (19), are minute dense particles of diameter 100 to 150 Å which are attached to the outer surfaces of the cytoplasmic vesicles. They are also scattered singly or in small groups in the surrounding cytoplasm (Fig. 3).

The degree of development and the distribution of the ergastoplasm vary widely in different types of cells. In cells that are known to be strongly basophilic such as the acinar cells of pancreas (Fig. 3) and plasma cells (Fig. 4) the development of the membrane system is very striking. In these cells a large proportion of the cytoplasm is filled with layers of flattened membranous vesicles or sacs covered with small granules.

Other cells in which the basophilia is less marked contain relatively few membranous structures and these usually take the form of round or irregularly shaped vesicles more or less covered with granules (Fig. 5).

However, in some cells known to be diffusely basophilic there are few vesicles but there are large numbers of ergastoplasmic granules scattered throughout the cytoplasm. This raises the question as to whether the RNA of the cytoplasm—the factor responsible for cytoplasmic basophilia—is actually in the walls of the sacs or in the granules attached to and scattered between the sacs. Palade (19) was the first to suggest that cytoplasmic basophilia is at least in part due to the granules. Our work with hepatoma cells (11), done soon afterwards, supported this conclusion because we found that the cytoplasm of the tumor cells had relatively few membranous sacs but many granules (Fig. 6). In stained sections the cytoplasm of these cells is diffusely basophilic. Thus there seemed to be a stronger correlation between the granules and the basophilia than between the membranes and the basophilia.

Recently Palade and Siekevitz (21) have shown by further experiments that it is very probable that the cytoplasmic RNA is confined almost entirely to the granules and is not present to any extent in the walls of the sacs themselves.

FIG. 2. Part of a normal rat liver cell showing a portion of the nucleus with a small nucleolus (*nu*). In the cytoplasm there are parallel arrays of elongated, flattened vesicles covered with ergastoplasmic granules (*er*), a small Golgi region (*Go*) at the middle right, and numerous mitochondria (*m*). A few dense membrane-bound bodies (*l*) and areas of glycogen (*gl*) are also present. (Magnification × 20,000)

FIG. 3. A small portion of the cytoplasm of an acinar cell from rat pancreas. The field shows an array of cytoplasmic vesicles or sacs, some round and others elongated. The vesicles contain a homogeneous substance of low density and are covered on their outer surfaces by numerous ergastoplasmic (RNA) granules. The granules are also present in the cytoplasm between the vesicles. (Magnification × 56,000)

FIG. 4. Part of a plasma cell from human bone marrow. A portion of the nucleus appears at the left. Most of the cytoplasm is filled with layers of membranous vesicles which appear rough-surfaced because of the attached ergastoplasmic granules. (Magnification × 30,000)

FIG. 5. Part of a lymphocyte from human bone marrow. The cytoplasm forms a thin rim around the nucleus. It contains a few mitochondria and in contrast to the plasma cell only scattered, small, round ergastoplasmic vesicles. (Magnification × 20,000)

FIG. 6. Parts of two adjacent cells from a Novikoff hepatoma. The mitochondria are smaller than those of normal liver cells (FIG. 2) and have well-developed cristae. Cytoplasmic vesicles are few in number but ergastoplasmic (RNA) granules are plentiful, being for the most part scattered about in the cytoplasm rather than attached to vesicles. The row of smooth-walled vesicles (lower middle) is probably derived from the adjacent cell membrane. Portions of two nuclei appear at the lower left and right. Areas of increased density, probably corresponding to the chromatin granules seen in the light microscope are visible in the nucleoplasm. The nuclear membrane is double-layered, the outer (cytoplasmic) layer showing occasional projections into the cytoplasm. (Magnification × 24,000)

FIG. 7. Striated or brush border of epithelial cell lining the small intestine of a rat. The brush border consists of microvilli which are finger-like extensions of the cytoplasm. The cell membrane forms a continuous covering over the microvilli. (Magnification × 64,000)

FIG. 8. Brush border of cell from proximal convoluted tubule of mouse kidney. The complex of projections and infoldings of the cell membrane which constitute the brush border has been cut at various angles. The cross-sectional appearance is shown at the lower right. (Magnification × 24,000)

PLATE I

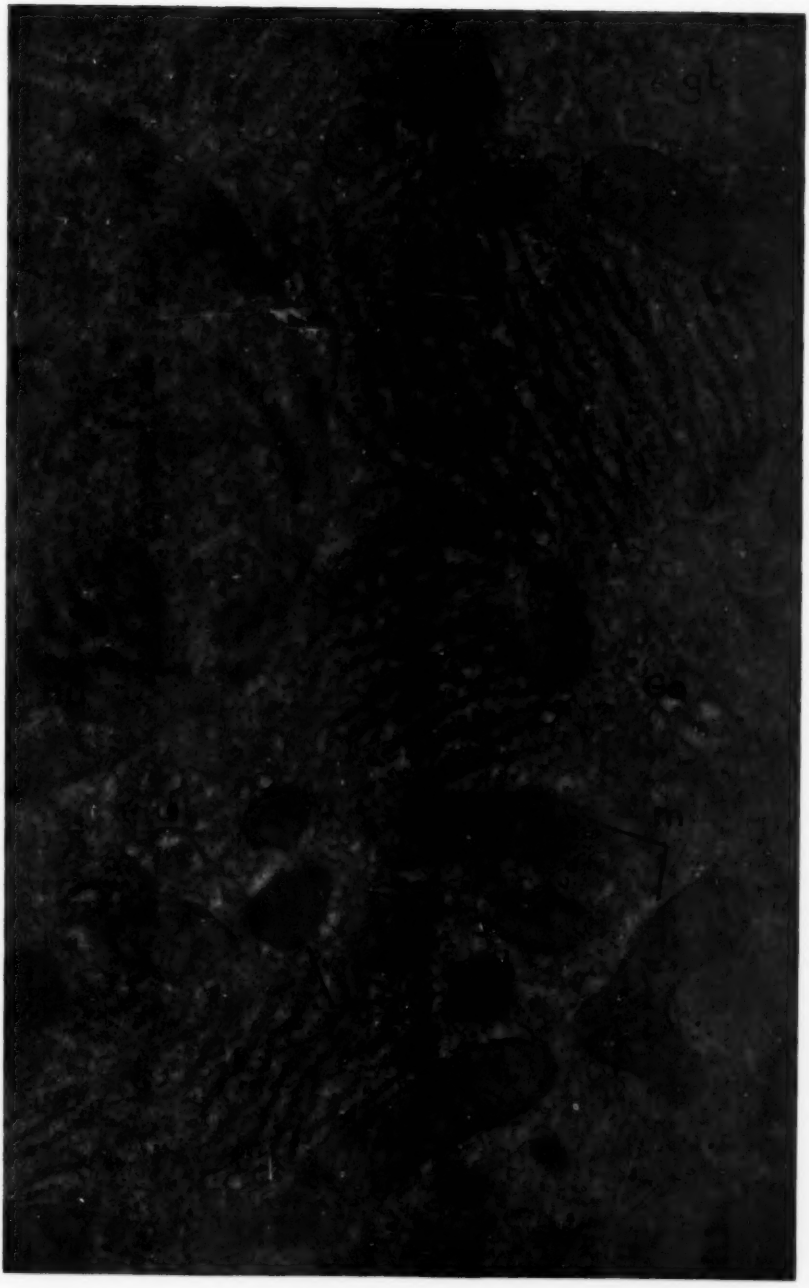

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PLATE II

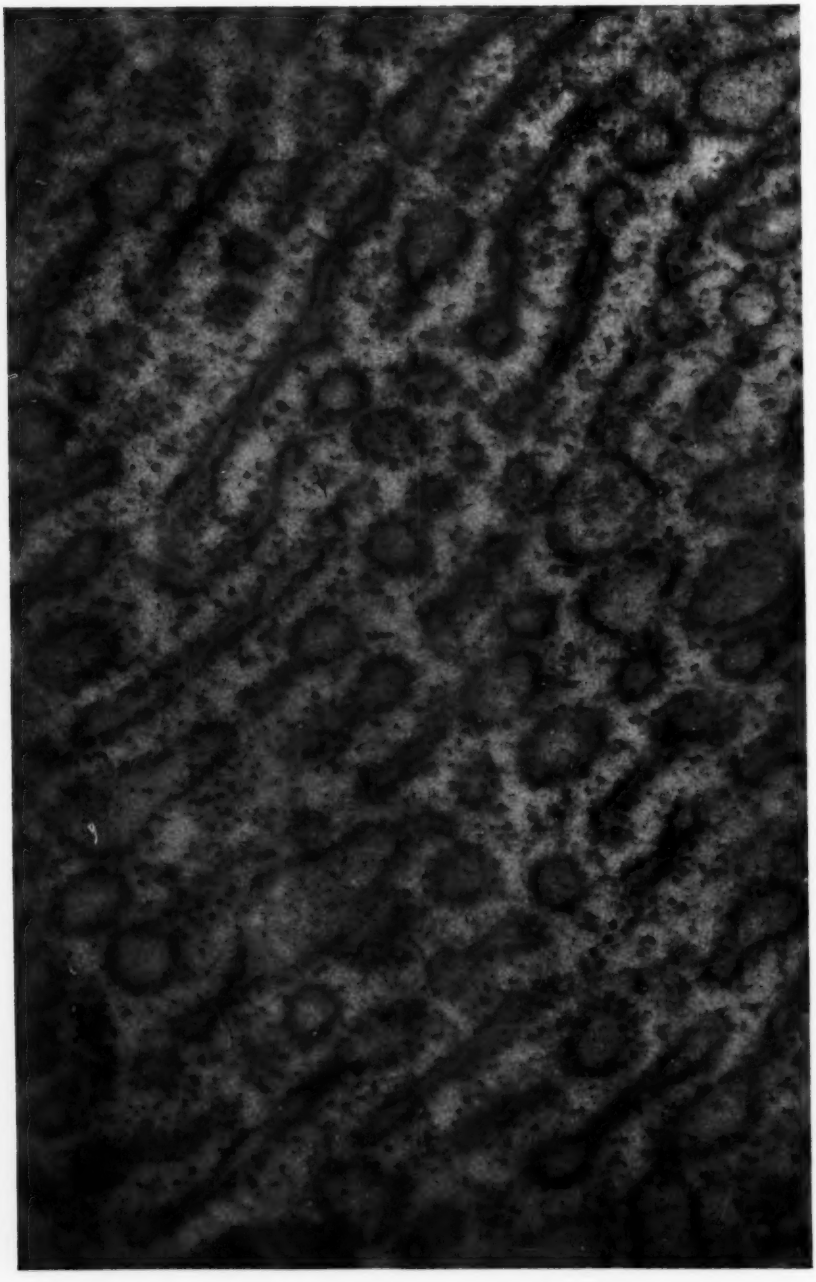

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PLATE III

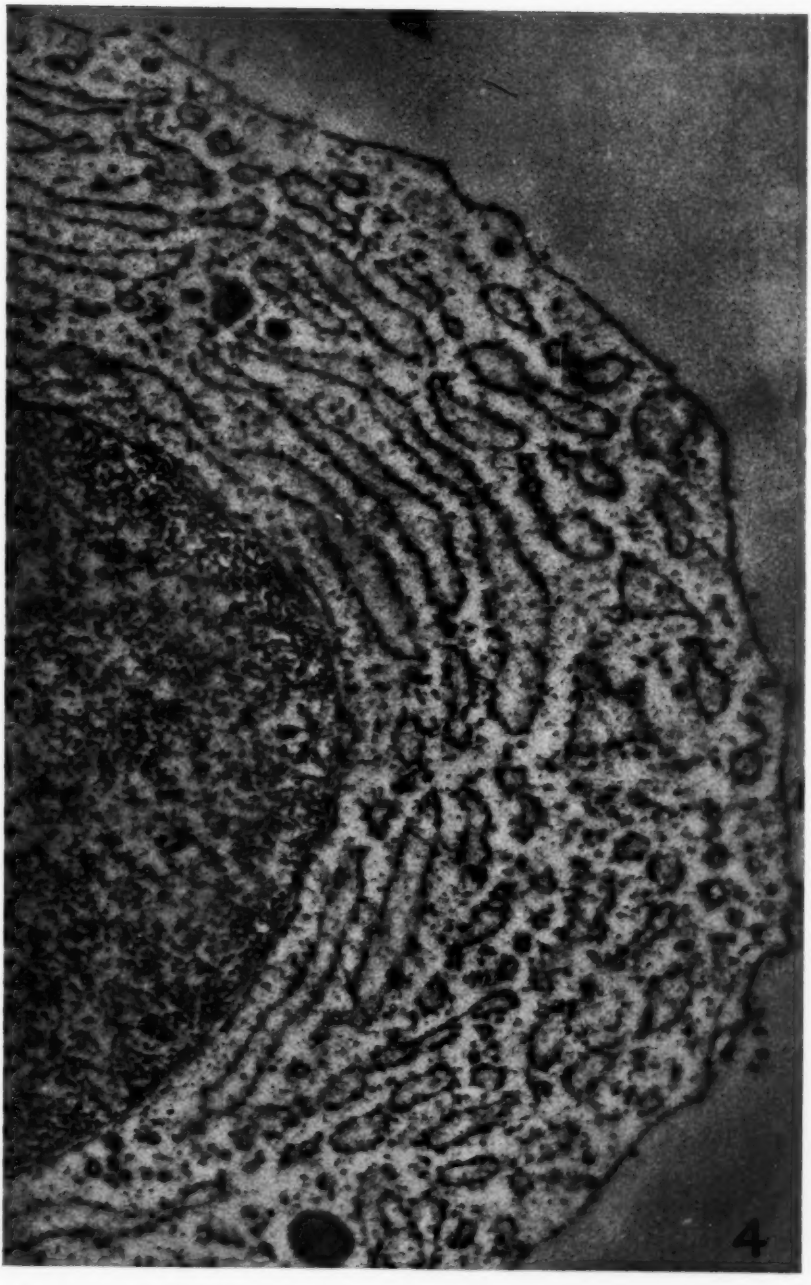

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PLATE IV

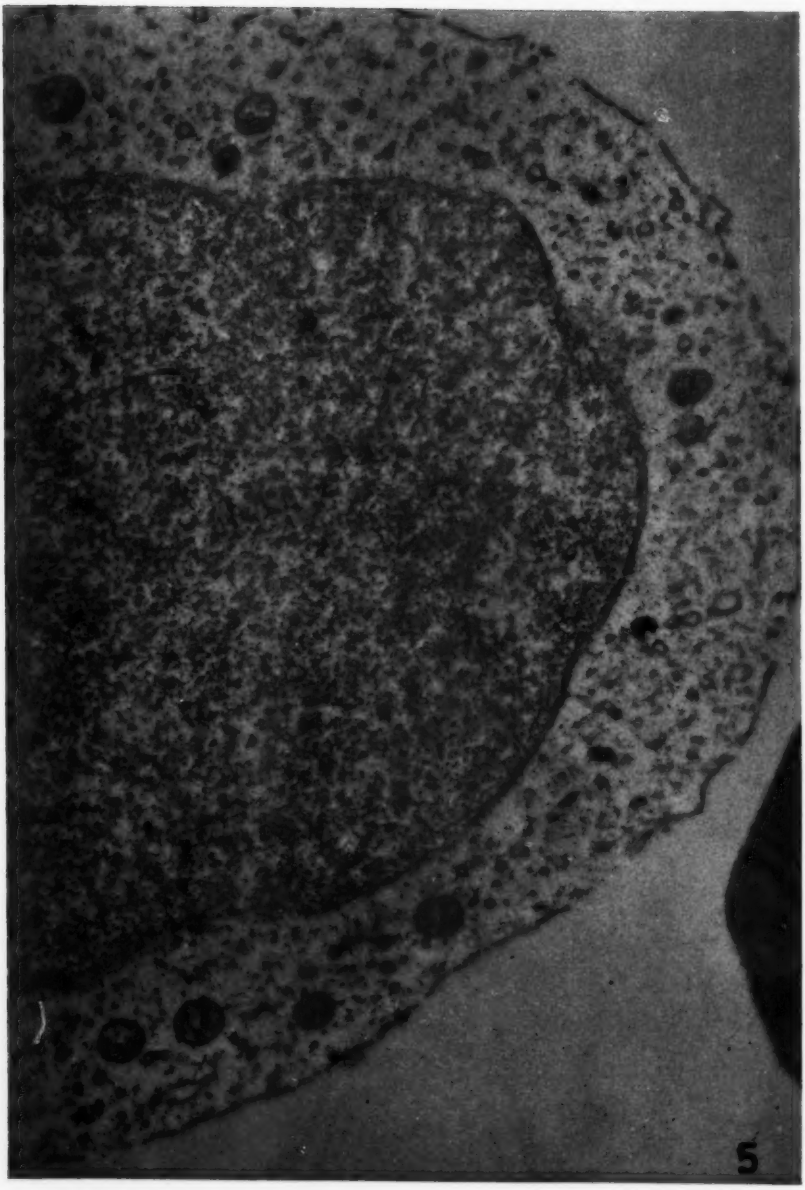

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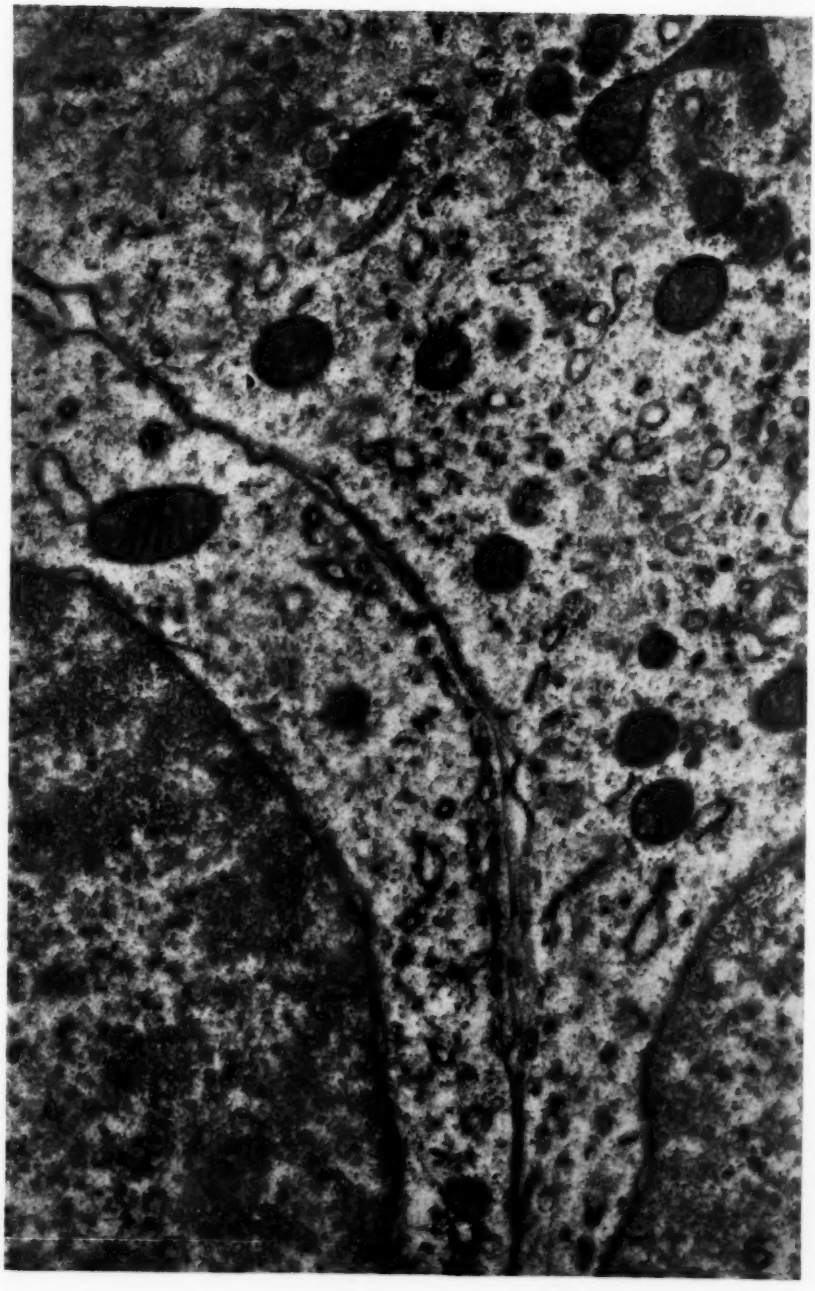
PLATE V



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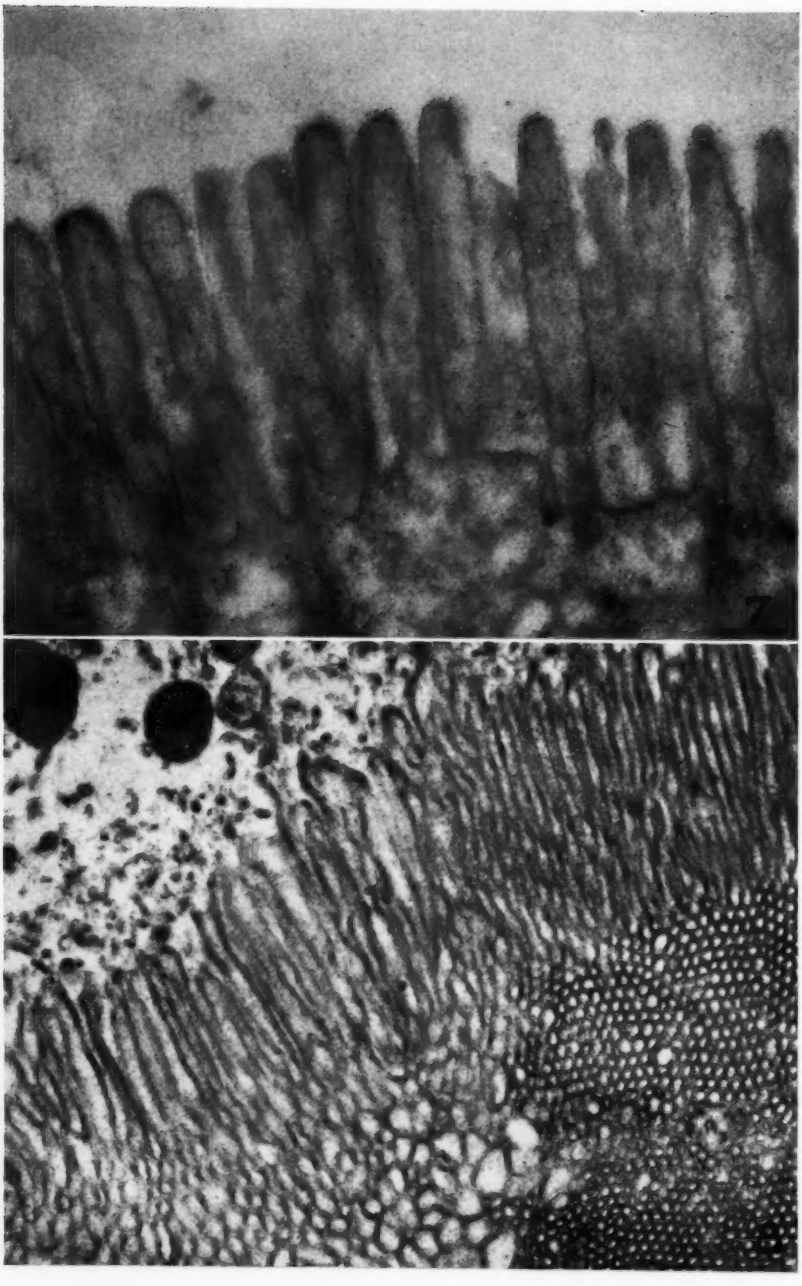

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Since ergastoplasm is identified with the light microscope by its staining reaction, and since it now appears that the RNA responsible for this staining reaction is contained in the small Palade granules which may or may not be attached to the membranous walls of the sacs, it would seem that the term ergastoplasm should strictly no longer be used for both granules and sacs but specifically for the granules. This may lead to the use of the term endoplasmic reticulum to refer to the systems of membranes that appear in the cytoplasm but since the membranous sacs or vesicles are not always arranged in a network, and since they are not always confined to the endoplasm this term is open to criticism. Indeed, as will be shown later, it seems best at the moment to describe the sacs and vesicles as comprising part of an extensive but not necessarily continuous system of cytoplasmic membranes extending from the cell membrane to the nuclear membrane. The other parts of this membrane system will now be considered.

The Cell Membrane and Its Specializations

The cell or plasma membrane usually appears in electron micrographs as a single dense line of width about 80 Å, though in a few sites it seems to be double-layered. Where cells adjoin one another the membrane is usually regular in outline but at a free surface it often appears convoluted. In some cells the convolutions are irregular in size and shape and are due to transitory movements of the mobile cell surface; in others their regularity indicates that they are a permanent feature of the cell surface. An example of the latter type may be seen in the cells lining the surface of the small intestine (Fig. 7). The striated border of these cells is seen in electron micrographs to consist of minute finger-like projections or microvilli, the cell membrane forming a continuous covering over the microvilli.

In other cells such as those of the proximal convoluted tubule of the kidney the brush border consists not only of projections but of deep invaginations as well. In cross section the invaginating cell membrane has a honeycomb appearance (Fig. 8). Other very complex interdigitations of the cell membrane are present in the basal portion of the cell where it adjoins the basement membrane. Similar appearances are seen in other types of cell, for example those of the choroid plexus (15). The function in all cases seems to be to increase the effective cell surface for absorptive purposes or fluid transport. These examples are mentioned here to show that the electron microscope has revealed that, in certain types of cell, the cell membrane is much more extensive and complex than was hitherto known or suspected.

The cell membrane and all the extensions of it which penetrate deeply into the cytoplasm are devoid of a coating of ergastoplasmic granules, and this distinguishes them from the granule-covered membranes of the cytoplasm which were described earlier. However, the two systems of membranes are not necessarily independent, as we shall now describe.

The invaginations which are a common feature of the cell surface are sometimes cut in such a way that they appear as vesicles in the cytoplasm

(Fig. 6). Like the cell membrane these vesicles have no attached granules. It is tempting to think that the membranous vesicles of the cytoplasm are formed by the ends of the invaginations being pinched off and subsequently moving towards the interior of the cell where they acquire the property of attracting the ergastoplasmic granules. Several investigators (11, 12, 19, 20, 25) have produced evidence to the effect that this process does occur though it is probably not the only way in which the cytoplasmic vesicles are formed.

The formation of vesicles from infoldings of the cell membrane resembles closely the process of pinocytosis. Pinocytosis refers to the absorption of nutriment or other fluid material by the cell in the form of comparatively large droplets rather than particles of molecular dimensions. It has been observed in living cells grown *in vitro* (14) and more recently has been studied with the electron microscope (17, 22). For example Palay and Karlin (22) studied the absorption of fat in the intestine and found that some of the fat is absorbed in the form of small droplets which pass down between the microvilli covering the cell surface into small depressions or invaginations which apparently deepen and eventually pinch off. The fat droplets then pass towards the interior of the cell still enclosed in membranous vesicles derived from the cell membrane. Bennet (2) has recently proposed a membrane flow hypothesis to explain such observations. It is clear that the existence of such processes has an important bearing on the question of cell membrane permeability.

In addition to the smooth-walled vesicles associated with the cell membrane there are, usually deep within the cytoplasm, other smooth-walled vesicles and membranes in closely packed arrays. The regions containing these structures have been identified with the Golgi region or Golgi apparatus, which is the next cytoplasmic component to be considered.

The Golgi Apparatus

In 1898 Camillio Golgi (9) described a structure within the cytoplasm of nerve cells that he named the internal reticular apparatus, and soon after similar structures were demonstrated in many different kinds of cells. The Golgi apparatus, as it came to be called, has been a subject of controversy among cytologists ever since. It is not necessary to go into the details of this controversy since it has become obvious that much of the confusion was due to the fact that most of the components of the Golgi complex are below the limit of resolution of the light microscope.

In the electron microscope the Golgi complex is seen to consist of three more or less distinct components, (*a*) a series of vacuoles that vary fairly widely in size and shape, (*b*) layers of closely packed membranes that border the vacuoles, and (*c*) numerous small round vesicles (microvesicles) scattered about in the adjacent cytoplasm. These are shown in diagrammatic form in Fig. 9. The fundamental structure seems to be the membranous layers which really consist of very elongated flattened vesicles or sacs easily distinguished from those described earlier in our discussion of ergastoplasm by the absence of the covering of Palade granules and by a much closer spacing

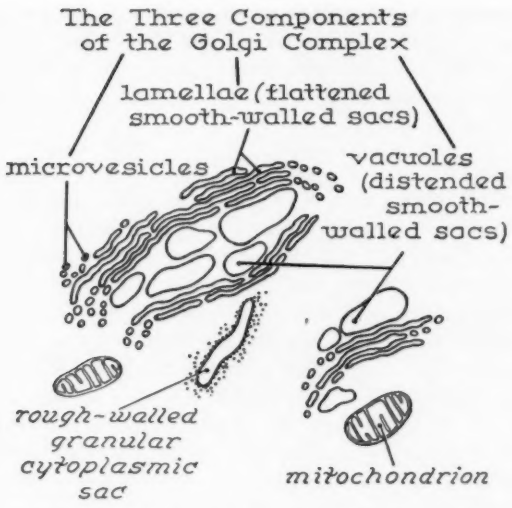


FIG. 9. Diagram of the Golgi complex, showing the three main components. The rough-walled (granular) cytoplasmic sac is not part of the complex but is shown for comparison with the smooth-walled sacs of the Golgi region. Two mitochondria are also shown.

between adjacent membranes. The vacuoles, which usually appear empty but occasionally contain some dense granular material, are simply distended portions of the sacs, and the microvesicles are probably small parts of the sacs that have become pinched off. The latter are smaller and more regular in shape than the rough-surfaced variety. They average about 400 Å in diameter in most cells.

The Golgi region is easily recognized in electron micrographs of all cell types by the features mentioned and no special treatment is required to demonstrate it. The region is usually clearly demarcated from the rest of the cytoplasm (Fig. 10), but occasionally, as in liver cells, it is more diffusely distributed (Fig. 2). As would be expected from the absence of RNA granules, the Golgi region does not stain with basic dyes.

Centrioles

Near the center of the Golgi region one or two dark dots may sometimes be seen with the light microscope; these are centrioles. Because of their scarcity and small size, centrioles are not often encountered in the thin sections used in electron microscopy. Bernhard and De Harven (3), however, have recently described the centriole as being in the form of an open-ended cylinder of width about 0.1 micron and length 0.3 to 0.6 micron. The wall of the cylinder is composed of a number (probably nine) of pairs of fibrils grouped around a central lumen (Figs. 11, 12). Between and around the fibrils, which are apparently hollow, there is a dense material.

Centrioles seem to be concerned in organizing fibrillar material; for example they probably play a part in the formation of cilia, the fine structure of which, it is interesting to note, bears some resemblance to that of centrioles. During mitosis, also, they are associated with the formation of the astral rays and the spindle.

Nuclear Membrane

The final membrane that we have to consider is the one that surrounds the nucleus. Although it is usually thought of as part of the nucleus there are good reasons for including it with the cytoplasmic membranes.

In electron micrographs the nuclear membrane is seen to be a double layered structure, with a total thickness of 300 to 400 Å. The outer layer often appears more tenuous than the inner. Recently a number of observers have noted the presence of pores of diameter around 500 Å in the nuclear membranes of many types of cell (Fig. 13). The outer and inner layers of the membrane are continuous at the pores. The pores would appear to allow practically unrestricted communication between the nucleus and the surrounding cytoplasm but the possibility of a very fine membrane covering the pore is not entirely excluded. The reality of these pores in living cells has been questioned but they certainly occur in cells which from other considerations appear to be in a very good state of preservation. The outer layer of the nuclear membrane carries on the cytoplasmic side numerous ergastoplasmic granules. Though the space between the two layers is fairly constant, sometimes small projections of the outer layer into the cytoplasm are seen. These projections are occasionally seen to be continuous with the rough-surfaced vesicles of the cytoplasm so that the nuclear membrane can be regarded as a specialized kind of cytoplasmic sac.

FIG. 10. Myelocyte from human bone marrow showing conspicuous Golgi region, well demarcated from the rest of the cytoplasm by the absence of mitochondria (*m*), specific granules (*s.g.*), and ergastoplasmic granules which are present throughout the rest of the cytoplasm. The horseshoe-shaped body (*c*) near the center of the Golgi region is a centriole. (Magnification × 16,000)

FIG. 11. An enlarged view of the centriole shown in FIG. 10, taken from an adjacent section. (Magnification × 40,000)

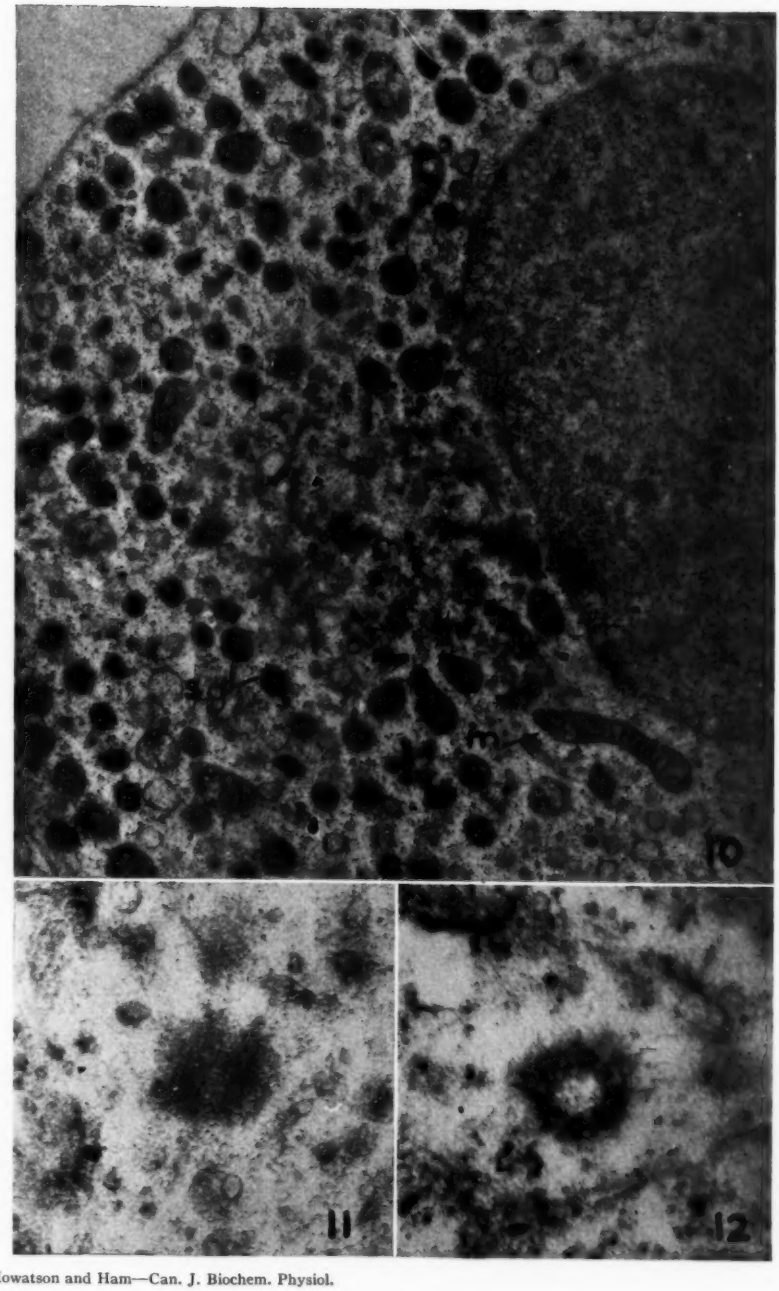
FIG. 12. Centriole from a chick embryo cell cut in cross section. (Magnification × 36,000)

FIG. 13. Novikoff hepatoma showing two pairs of adjacent cell membranes (*c.m.*) and parts of two nuclear membranes (*n.m.*). Pores in the nuclear membranes are indicated by arrows. (Magnification × 27,500)

FIG. 14. Junction of two cells from monkey heart muscle showing dense material on the inner aspects of the cell membranes. These layers of dense material constitute the 'intercalated discs' of heart muscle. (Magnification × 36,000)

FIG. 15. Mitochondrion from a hepatoma cell showing the double-layered enveloping membrane and a series of parallel double-layered internal membranes. At the points marked by arrows the internal membranes are seen to be continuous with the inner layer of the enveloping membrane. (Magnification × 104,000)

FIG. 16. Portion of the cytoplasm of a liver cell from a rat which had been starved for 4 days, for comparison with a normal rat liver cell (FIG. 2). The mitochondria are markedly swollen but retain the short, poorly developed cristae characteristic of normal liver cell mitochondria. Dense bodies (*l*) distinct from mitochondria and similar to those shown in FIG. 2 are visible. The parallel arrays of cytoplasmic vesicles seen in normal liver cells appear to have broken down into small irregular vesicular elements. (Magnification × 20,000)



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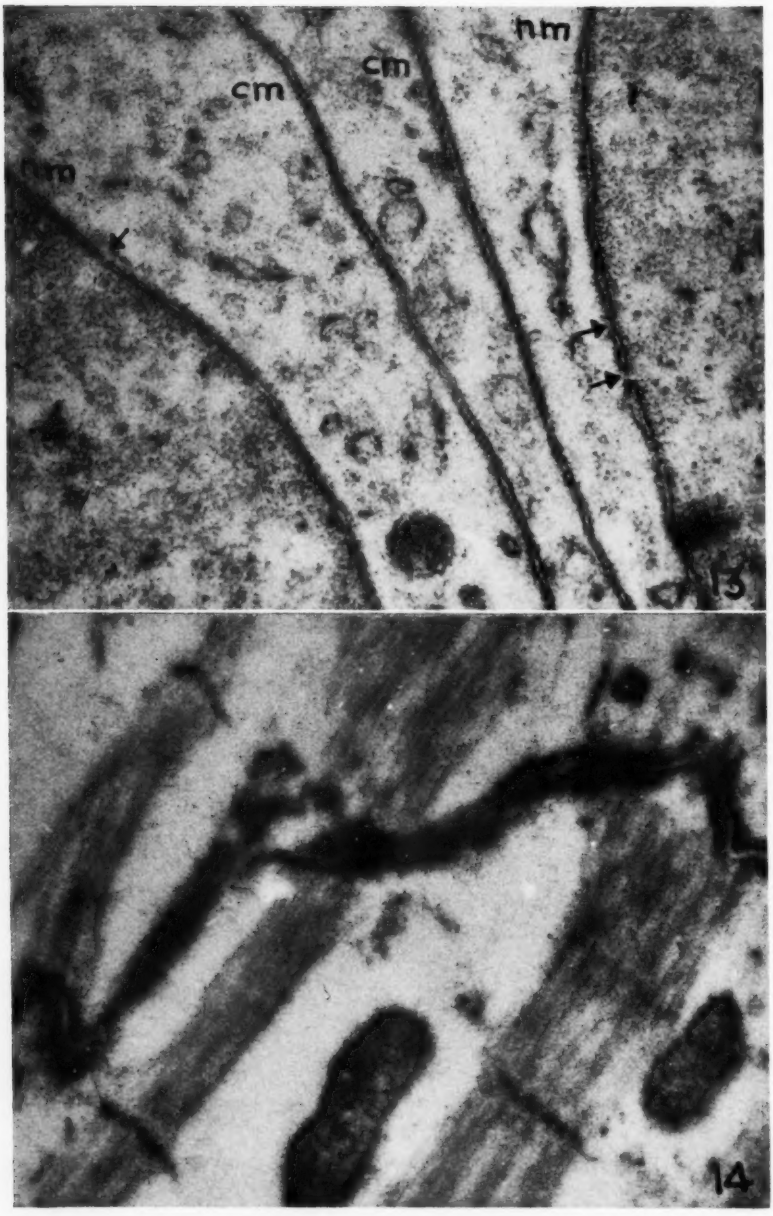

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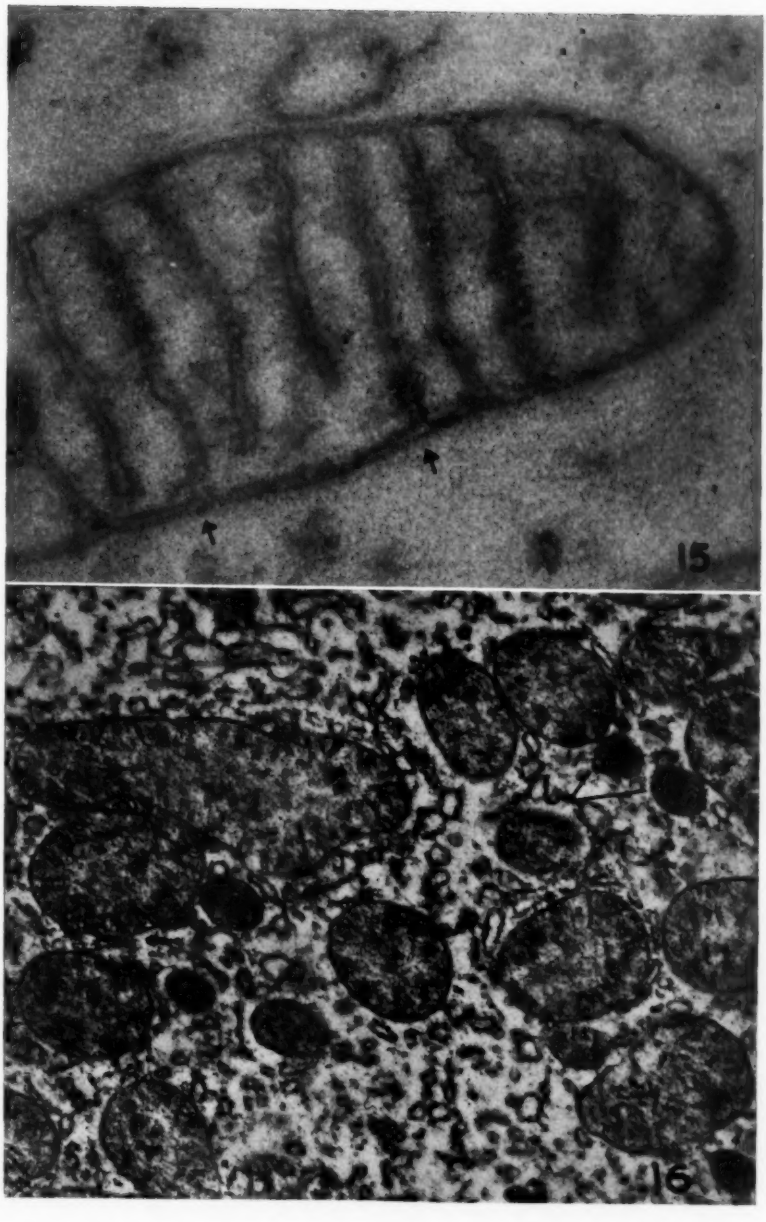
PLATE IX



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We have now described four different membrane systems; the cell membrane and its specializations, the system of membranes with which the ergastoplasmic granules are associated, the smooth membranes of the Golgi region, and the nuclear membrane. The presently available evidence suggests that all these membranes are part of one complex membrane system which is differentiated in different parts of the cell to perform different functions. These functions will now be briefly discussed.

Functions of the Cytoplasmic Membrane Systems

The cell membrane, as we have seen, is often specialized in such a way as to facilitate fluid transport. Another function of the cell membrane is to bind adjacent cells together. The way this is accomplished is not fully understood. In electron micrographs contiguous cell membranes may be seen to follow parallel courses for long distances with a separation of only about 200 Å, there being no indication of any binding or cement substance between the membranes (Fig. 13). In certain types of cells, however, there are areas close to adjacent membranes where the cytoplasm is of increased density. These seem to be regions where especially firm contact is established between cells. This type of structure reaches its highest state of development in heart muscle where strong contact between cells is essential. In electron micrographs it is seen that the intercalated discs of heart muscle consist of aggregations of dense material along the borders of adjacent cells (Fig. 14).

The most extensive part of the cytoplasmic membrane system is usually that with which the ergastoplasmic granules are associated. These granules consist largely of RNA and there is good evidence to the effect that RNA plays an important part in protein production. It is therefore significant that cells in which this membrane system is highly developed are ones that are synthesizing special protein secretions. Examples of this which we have already mentioned are pancreatic acinar cells which secrete digestive enzymes and plasma cells which produce the protein globulins. A widely held view is that the seat of the production of the special proteins is on the cytoplasmic membranes, which have the attached RNA granules.

Cells in rapid growth such as embryonic or tumor cells do not as a rule have an extensive cytoplasmic membrane system, although they must be producing much protein. They do, however, have a large complement of unattached RNA or ergastoplasmic granules. It would seem therefore that unattached granules are concerned with general protein production for purposes of growth whereas granules that are part of an organized membrane system are engaged in the synthesis of special proteins for secretions.

Mitochondria

The cell components so far considered are dispersed to a greater or less extent throughout the cytoplasm and may in some instances be interconnected. The next organelles to be discussed, the mitochondria, however, form distinct structural units within the cytoplasm. They can be separated from other cytoplasmic material in fairly pure form, and consequently their biochemical

properties can be determined relatively easily. For this reason more information has been obtained about mitochondria than about any other cell organelle. It is known, for example, that mitochondria contain several complex enzyme systems. The activities of the various enzymes are well coordinated, suggesting that there must be a considerable amount of structural organization within each mitochondrion. No details of internal structure are visible with the light microscope. However, when it became possible to examine mitochondria in thin sections with the electron microscope, a complex internal structure was revealed within them.

Each mitochondrion is surrounded by a double-layered enveloping membrane and contains within it a series of double-layered membranes, arranged transversely to the long axis of the mitochondrion and roughly parallel to one another (Fig. 15). The dense layers of the membranes are each about 50 Å thick and the clear space separating the layers is about 80 Å in thickness. The mitochondrial matrix is usually denser than the surrounding cytoplasm and appears structureless except for the presence of a few densely

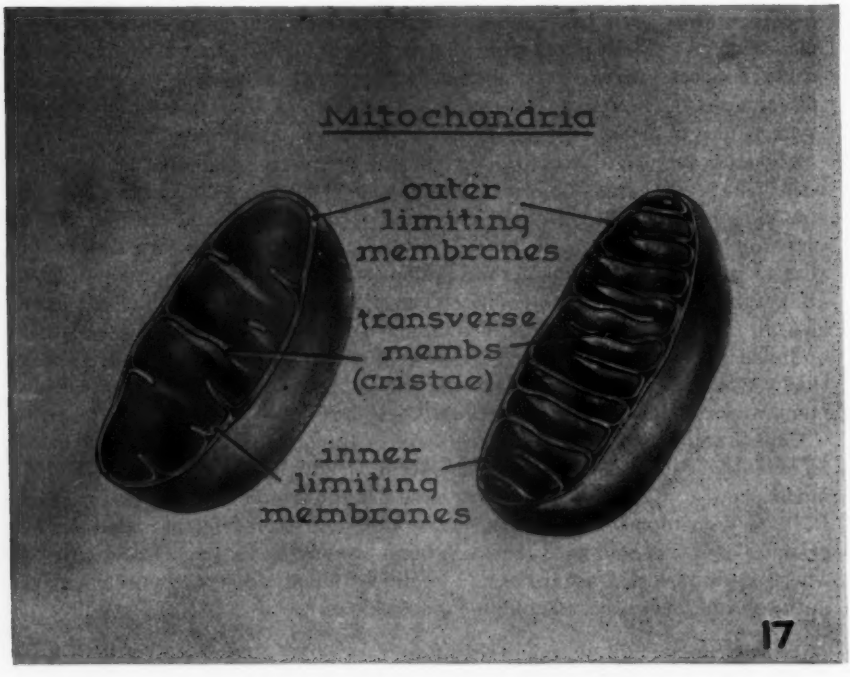

FIG. 17. Diagram showing the three-dimensional structure of mitochondria. In the mitochondrion on the left, the internal membranes extend only part way across the organelle. This is typical of liver mitochondria. A commoner arrangement is shown on the right where many of the membranes extend completely or almost completely across the interior of the mitochondrion. The double-layered enveloping membranes are also shown.

staining granules in the mitochondria of certain cell types. This arrangement of transverse and enveloping membranes is of remarkably widespread occurrence, being present with only minor variations in the mitochondria of all kinds of animal cells so far examined.

The three-dimensional appearance of typical mitochondria deduced from a study of a number of cross-sectional views in micrographs is shown in Fig. 17. Most investigators describe the transverse membranes as infoldings of the inner layer of the enveloping membrane as they are depicted in Fig. 17. To these infoldings Palade (18) has given the name 'cristae mitochondriales'. The cristae extend for varying distances into the lumen of the mitochondrion, but according to Palade they never completely divide the mitochondrion into separate compartments.

However, the degree of development of the membranes varies considerably from one cell type to another. In mitochondria from mature rat liver (Figs. 2, 16) for example, the internal membranes are poorly developed and seldom extend as far as halfway across the organelle. This type is illustrated on the left in Fig. 17. In many other types of cells the majority of sectioned mitochondria have well-developed transverse membranes (Figs. 6, 10). A three-dimensional reconstruction of this type is shown on the right in Fig. 17.

It has been noted that mitochondria differ in the details of their structure from one cell type to another. They may also differ in the same type of cell under different physiological conditions. For example in starved animals mitochondria in liver cells are larger and rounder than normal liver mitochondria (Fig. 16).

Even more striking differences are observed if we compare the mitochondria in normal liver cells with those in the hepatomas that we have studied (11). In the hepatoma cells mitochondria are less numerous and on the average smaller than in normal liver cells, though the range in size is considerable. Furthermore the transverse membranes are much more highly developed in that they extend much farther across the mitochondria than their normal counterparts (Figs. 6, 15).

Fractionation of Cytoplasmic Components

We have already mentioned that mitochondria can be isolated in fairly pure form. This is achieved by differential centrifugation of cell homogenates prepared as a rule in sucrose solutions. The method of thin sectioning has recently been used to examine the purity of the mitochondrial fractions obtained in this way and also the morphological integrity of the mitochondria (13, 26). These two factors are of considerable importance in evaluating the biochemical information obtained by studying the isolated material. It can be seen (Fig. 18) that mitochondria isolated by standard procedures from rat liver are reasonably well preserved morphologically; they can easily be recognized by their characteristic features. The fraction contains, in addition to mitochondria, a small proportion of vesicular elements, derived from the cytoplasmic membrane systems.

By using higher centrifugal forces, it is possible to separate fractions containing components smaller than mitochondria. Claude (5) was the first to isolate from homogenates of liver and other tissues a fraction consisting of what appeared to be small particles, which he called microsomes. The fraction containing them came to be known as the microsome fraction. This fraction was rich in RNA, suggesting that it was derived from the basophilic component of the cytoplasm. However, the particles were of a size below the limit of resolution of the light microscope so it was not certain at that time what their morphological counterpart was in the intact cell. It was assumed that microsomes were something that actually existed as such in cells. When it became possible to examine sections of fractions in the electron microscope it was realized that the so-called microsomes were for the most part bits and pieces resulting from the breaking up of large membranous sacs of the cytoplasm during homogenization. The smaller membranous sacs associated with the cell membrane and the Golgi region also contributed to a lesser extent to the microsome fraction (Fig. 19).

Other Cytoplasmic Constituents

Certain types of cells contain other cytoplasmic constituents which we have time to mention only briefly. In recent biochemical investigations evidence has been found for the existence in liver fractions of particles somewhat smaller than mitochondria and functionally different from them (1, 16). These particles, sometimes called lysosomes, may correspond to dense membrane-bound bodies often seen in liver cells (Figs. 2, 16). The enveloping membrane is single-layered and the bodies usually contain irregular areas of increased density. Somewhat similar bodies, morphologically distinct from mitochondria, are seen also in other types of tissue (10).

In another category are the specific granules of leukocytes (Fig. 10) and the secretory granules of secretory cells. Fine fibrils are present in the cytoplasm of some cells and are particularly well developed in muscle cells (Fig. 20).

FIG. 18. Mitochondrial fraction isolated from a homogenate of normal rat liver in 0.88 *M* sucrose by differential centrifugation. The mitochondria are fairly well preserved and easily recognizable. Fragments of cytoplasmic membranes are present as contaminants. (Magnification × 20,000)

FIG. 19. Microsome fraction from a homogenate of Novikoff hepatoma cells. It consists mainly of vesicles with attached ergastoplasmic granules and groups of unattached granules. (Magnification × 20,000)

FIG. 20. Part of an immature muscle cell from a chick embryo. A small portion of the nucleus is shown at the upper right. In the cytoplasm a developing muscle fibril is seen at the left. Two adjacent *Z* bands with myofilaments stretching between them are visible. (Magnification × 43,500)

FIG. 21. Nucleolus from a hepatoma cell. The main constituent is a coiled filament which consists of closely packed uniform granules similar to the RNA granules of the cytoplasm. In the surrounding nucleoplasm the granules are less closely packed and less uniform in size so that although there is no nucleolar membrane the limits of the nucleolus are fairly distinct. (Magnification × 27,000)

FIG. 22. Myelocyte from human bone marrow in metaphase. The chromosomes appear as irregularly shaped, dense granular masses near the center of the figure. At the upper center can be seen traces of spindle formation, the elements of the spindle radiating towards the chromosomes. (Magnification × 12,000)

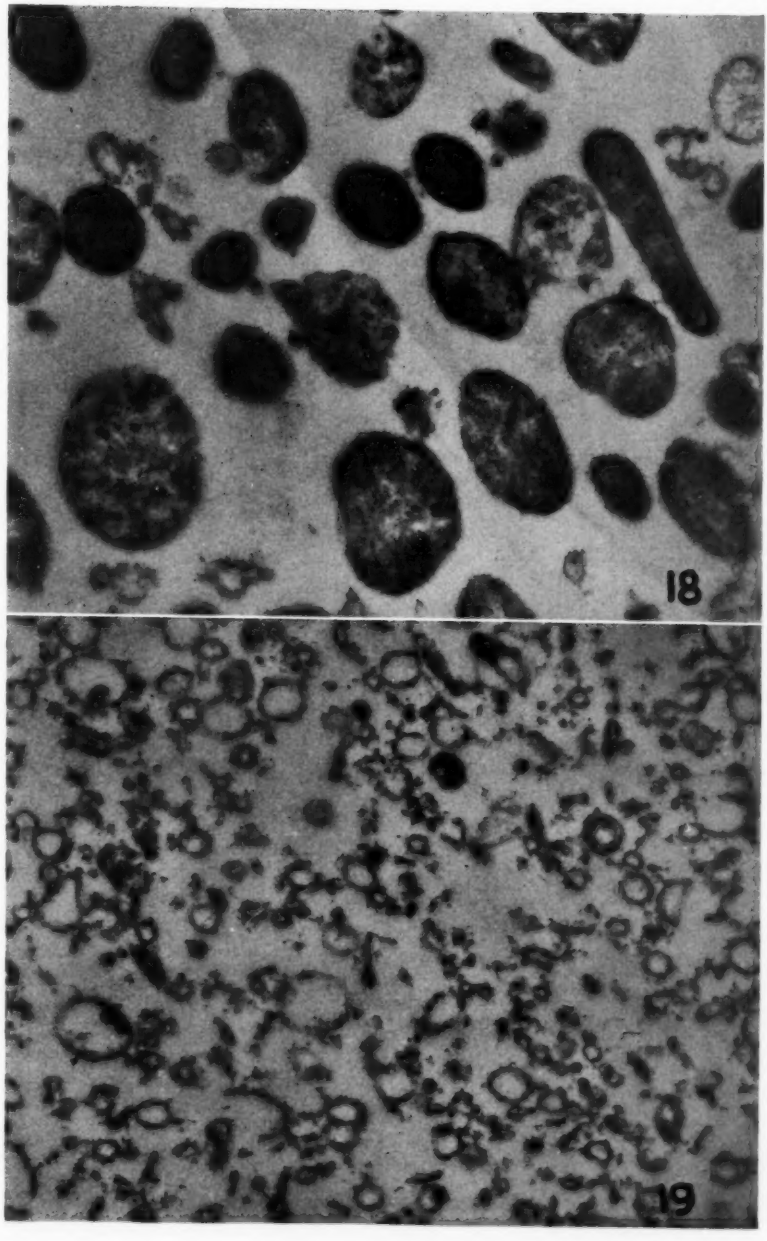

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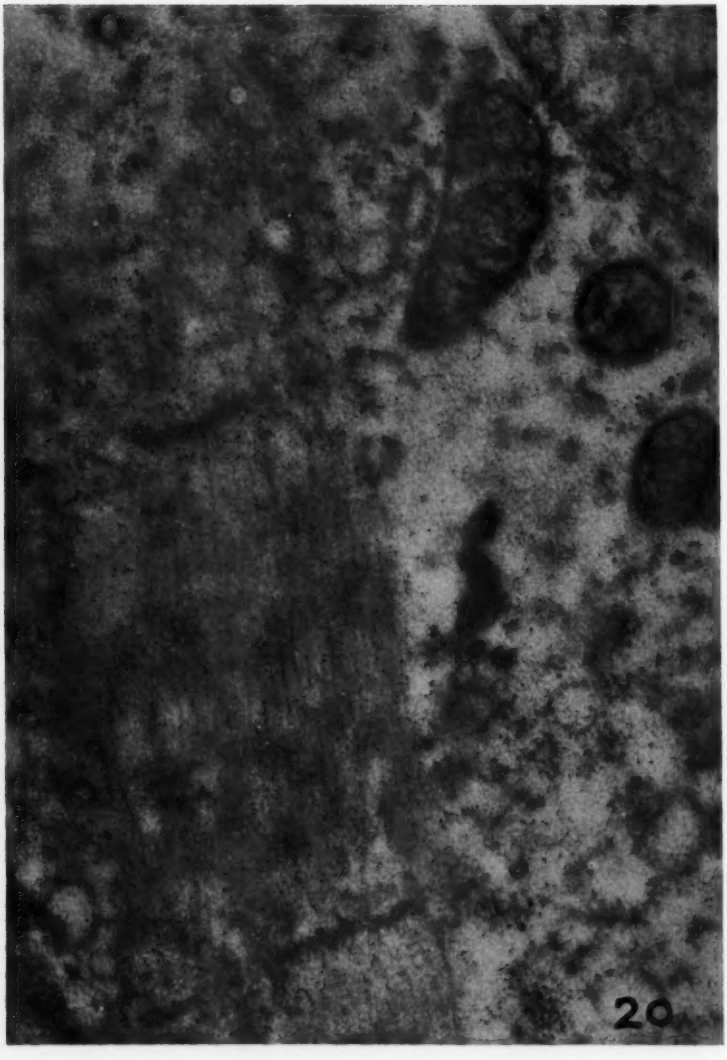
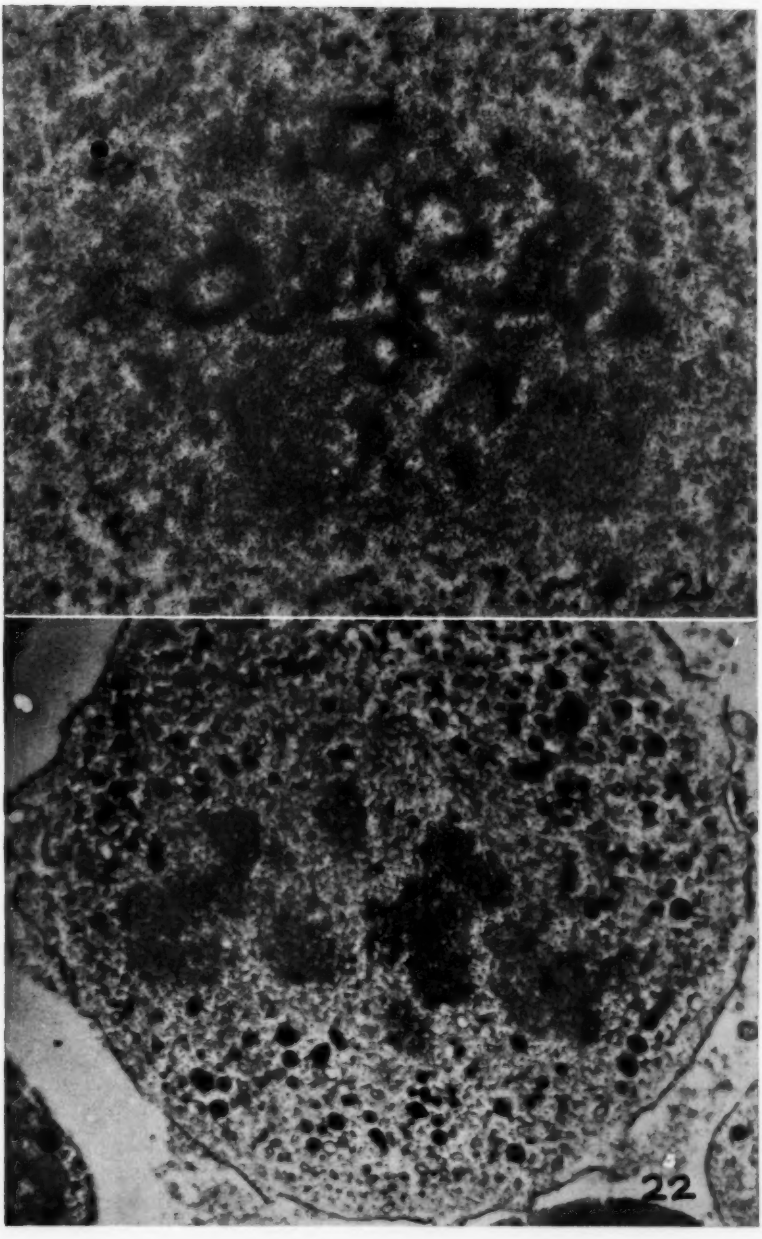
PLATE XI





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The Nucleus

The thin sectioning methods in electron microscopy that have led to a considerable increase in our knowledge of cytoplasmic structure have not been so informative about the nucleus, although they have been of some help in elucidating certain structures, e.g. that of the nucleolus, as will now be described.

The main constituent of the nucleolus is a system of coiled filaments; these are embedded in a homogeneous material. The coiled structures have been named the *nucleolonema* and the homogeneous part the *pars amorpha* by Estable and his associates (7), who studied nucleoli with the light microscope. By electron microscopy it has been shown that the coiled filaments are made up of small dense granules about 100–150 Å in diameter (Fig. 21). These resemble in size and density the ergastoplasmic granules of the cytoplasm. There is no suggestion of a membrane surrounding the nucleolus.

The rest of the material of the nucleus usually presents a fairly homogeneous granular appearance but the granules are coarser, less uniform in size, and less closely packed than those of the nucleolus. In some cells denser regions probably corresponding roughly to the chromatin granules observed in light microscopy may be seen, particularly close to the nuclear membrane (Fig. 6).

It is generally believed that in the interphase nucleus the chromonemata of the chromosomes are in the form of hydrated, loosely coiled filaments in contrast to their form in the mitotic nucleus when they become relatively dehydrated and more tightly coiled, and so stainable. In the interphase nucleus only heterochromatic regions along chromosomes stain well enough to give rise to the appearance of chromatin granules. It has been suggested that these stainable granules may represent longitudinal portions of chromosomes which are more tightly coiled than other parts, and so dense enough to stain.

If the bulk of the structure of an interphase nucleus consists of loosely coiled, extremely fine threads, it is obvious that in the very thin sections used in electron microscopy the individual coils of the threads would always be cut in cross and oblique section and therefore the threads would appear in sections as tiny round dots, or as very short rods. Rows of such dots are sometimes seen in arrangements that suggest that a coiled structure has been sectioned longitudinally for a short distance. Accordingly the electron microscopy of the interphase nucleus has not revealed anything that does not fit in with the structure that it has been assumed to possess from light microscopic studies.

The Mitotic Nucleus

Very little new information has so far been obtained by electron microscopy about the mitotic nucleus. Because of the limited area scanned in the electron microscope, mitotic figures are seen rather infrequently even in rapidly growing tissue. Unless the formidable task of cutting serial sections is undertaken, information has to be pieced together from incomplete two-dimensional pictures of the process. A thorough study of the mitotic process

by electron microscopy has not yet been done to our knowledge but it would undoubtedly be of great value.

Fig. 22 shows a human bone marrow cell in metaphase. The chromosomes appear as irregularly shaped objects with little evidence of internal structure. The spindle is represented by a series of thin filaments stretching from the chromosomes towards a point in the cytoplasm.

In conclusion, although the electron microscope has been applied effectively to the study of cell structures for only a very few years, it has already revealed information of fundamental importance to the understanding of cell processes, and much more can be expected of it in the future.

Further Comments in Answer to Questions

With regard to the technique of cutting the sections, there are three main requirements: (1) a microtome capable of regularly advancing the block by steps of a few hundred Å between each cutting stroke (this has been achieved both by a mechanical and by a thermal type of advance mechanism); (2) a suitable cutting edge (the edge most commonly used is the fracture edge of a piece of plate glass but Fernandez-Moran has recently achieved very good results using specially polished diamond edges which last much longer than glass and can be used to cut harder materials); (3) an embedding medium with satisfactory cutting properties (the medium almost universally used in this type of work is methacrylate resin formed by polymerizing the liquid monomer with which the fixed and dehydrated tissue specimen is previously impregnated).

On the question of the continuity of the vesicular structures seen within the cytoplasm there is some difference of opinion. The term 'endoplasmic reticulum' was introduced by Porter and Kallman because their earlier work indicated that all the vesicular structures were connected together. This is probably true in some cells but in other cells it is almost impossible to believe that the vesicles can be connected because they are so far separated from one another. Undoubtedly, however, some of the vesicles that appear separate in the thin sections are really portions of tubules or other continuous structures.

The first evidence for the existence in liver of cytoplasmic particles with properties different from those of either mitochondria or microsomes was obtained by de Duve and his colleagues from biochemical analysis of liver fractions. Drs. Allard and de Lamirande have recently been working in this field. They have shown that of nine fractions isolated from rat liver in 0.88 *M* sucrose, the intermediate fractions 5, 6, and 7 contain most of the uricase and acid phosphatase whereas fractions consisting mainly of mitochondrial or microsomal elements contain other enzymes but very small amounts of uricase and acid phosphatase. This would confirm the presence in the intermediate fractions of elements of other than mitochondrial or microsomal origin.

Regarding the size and appearance of mitochondria it is clear that there are considerable variations from one cell type to another, and less marked variations within a given cell type. Changes in morphology in response to physiological changes have been noted (as, for example, the swelling of the mitochondria in the livers of starved rats) but so far as I know the size and density of mitochondria have not been related to possible secretory activities of the organelles.

The study of pathological material has to be preceded by a thorough investigation of normal materials, and so far very little new information has been obtained even about the normal structure of chromosomes. They reveal a disappointingly small amount of organized structure; this is no doubt due to the fact that we are dealing with spiral structures which are not easy to interpret when seen in thin sections since they appear as series of dots or short rods. Recently Fawcett and others have shown that meiotic chromosomes in prophase have a definite central cord which seems to be a spiral structure. This apparently disappears at metaphase but it must have some significance and offers hope that in the future we will get to know more about the structure of chromosomes.

Acknowledgments

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DISCUSSION: M. L. Barr[1]

Dr. Howatson's presentation has demonstrated clearly the contributions of electron microscopy in advancing our knowledge of the ultrastructure of the cell. Current methods of electron microscopy are especially productive in resolving the fine structure of cytoplasmic constituents, in particular the mitochondria and the endoplasmic reticulum. In general, study of the nucleus has been less rewarding, although technical refinements are overcoming some of the difficulties. Considerable headway has been made in studying the fine structure of the nuclear membrane and, to a lesser degree, the nucleolus. Less information has accrued on the structure of chromosomes in dividing cells, but informative papers on this subject are now appearing. The study of the structure of the chromatin and related components of the intermitotic nucleus has proved especially difficult. Since Dr. Howatson was obliged to deal with the ultrastructure of the nucleus briefly, a certain aspect of nuclear structure will be discussed. Attention is drawn to the possibility of somatic pairing of chromosomes, since this is a morphological detail that could have an important bearing on several aspects of biology and medicine. Although electron microscopy is not involved, the subject of somatic pairing of chromosomes is not far removed from the general field of cellular ultrastructure.

The point at issue is whether the two members of a pair of chromosomes, one of paternal and the other of maternal origin, lie side-by-side in the resting nuclei of body cells generally,

[1]Contribution from the Department of Microscopic Anatomy, University of Western Ontario, London, Ontario.

as they do in certain stages of meiosis in germ cells. Such an arrangement is not included in the current concept of the distribution of chromosomes in resting nuclei of mammalian cells. The importance of somatic pairing is that it provides a possible basis for the redistribution of genetic factors during mitosis and could, therefore, have a bearing on normal and abnormal differentiation of cells and tissues.

Suspicion that somatic pairing of chromosomes may occur in mammalian cells arises from an item of observation in connection with the sex chromatin. On careful examination of favorable material, the female sex chromatin can be resolved into two components. The bipartite nature of the sex chromatin has been seen clearly in several mammals, including man. Because of this observation, and for other reasons, the female sex chromatin is thought to represent heterochromatic portions of the two *X* chromosomes, i.e., portions that remain compact and deeply staining in the resting nucleus. Although euchromatic regions of the *X* chromosomes cannot be distinguished in resting nuclei, one is entitled to suspect that they are also closely related spatially, and that the autosomes may be arranged similarly in synaptic pairs.

The foregoing suggestion, although novel for mammalian cells, loses a good deal of its originality when reference is made to observations on plants and lower animals. Over 20 years ago, Watkins (5) gathered from the literature references to somatic pairing of chromosomes in 33 species of plants. Known examples of somatic pairing are even more numerous in insects, this chromosomal arrangement having been described for upwards of 100 species. This subject has received little attention in vertebrates, but two examples are on record. Somatic pairing has been described in the newt by Boss (1) and in normal cells of the frog and in an adenocarcinoma of the frog's kidney by Duryee and Doherty (2).

If the matter were simply a question of whether homologous chromosomes lie side-by-side in pairs in the resting nucleus the subject would have little interest except from the strictly morphological point of view. The potential importance of somatic chromosomal pairing lies in the possibility of crossing over or exchange of chromosomal material during mitosis, similar to that which occurs during chromosomal synapsis in gametogenesis. Stern (4) has, in fact, described crossing over between homologous chromosomes in somatic cells of *Drosophila*, and Huskins (3) has noted chiasmata, or the connections between chromosomes that precede the exchange of chromosomal segments, in plant cells.

Although it has to be determined by direct observation whether crossing over occurs in mammalian somatic cells, the following sequence of events is theoretically possible. A pair of chromosomes may be visualized in synapsis, one chromosome bearing the dominant alleles *A* and *B* toward either extremity, and the other chromosome bearing the recessive alleles *a* and *b* in the same locations. A tetrad is formed by the duplication of each chromosome in early prophase. A chiasma may now be visualized as occurring between two members of the tetrad about halfway along their lengths, followed by an exchange of chromosomal material. If the chromosomes are now followed through mitosis, it will be found that the daughter cells will, in certain instances, lack the dominant allele for one of the genes. The genetic properties of such a daughter cell would be different from those of the parent cell for the gene in question. There is, therefore, in somatic pairing of chromosomes the possibility of effecting altered genetic properties of cells during mitotic division.

To conclude these speculative comments on the cell nucleus, four questions are asked.

(i) Are homologous chromosomes paired in somatic cells, in accordance with the evidence for somatic pairing of the *X* chromosomes that arises from observations on the bipartite nature of the female sex chromatin ?

(ii) Does crossing over or exchange of chromosomal material between homologous chromosomes occur in somatic cells as it does in germ cells ?

(iii) If crossing over does occur, do altered genetic properties that arise during the course of mitotic divisions have a bearing on the differentiation of cells and tissues during embryological development ?

(iv) Since carcinogens are known to alter the physical properties of chromosomes, may such agents operate in part by inducing unusual genetic properties in somatic cells by interfering with normal mechanisms of somatic pairing, chiasma formation, and exchange of chromosomal segments ?

These are large questions and finding answers to them will be a long and difficult task. Although the suggestions offered in this discussion are largely hypothetical, it is felt that the possible importance of somatic pairing of chromosomes justifies introducing the subject in this Symposium.

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ON THE FINE STRUCTURE OF MICROBES

A SUMMARY[1]

R. G. E. MURRAY

This brief diversion of the main stream of discussion towards microbial cells is justified mainly by the great success that has attended the use of bacteria and fungi, in particular, as convenient test objects for exploring the metabolic machinery of living cells. This fruitful approach has lent support to a general article of faith that the fundamental processes of all living cells are very similar. It is easy to allow this sort of generalization to prevent the recognition of those characters that make a kind of cell unique among all kinds of living cells. For the mind must be ". . . nimble and versatile enough to catch the resemblances of things (which is the chief point), and at the same time steady enough to fix and distinguish their subtler differences . . ." (3). This argument has been very neatly and provocatively presented by Stanier (24) in an essay entitled *Some Singular Features of Bacteria as Dynamic Systems*; it provides a most appropriate introduction to this contribution, which is mainly concerned with the unique features of bacterial structure as revealed by the electron microscope.

There are very few electron microscope studies, so far, on which to base generalizations about the fine structure of either the protozoa or the fungi. The protozoa show a fascinating complexity of fine structure scarcely to be equalled in any other group (see 7, 16, 19, and 21). Yet, the appearance of the cytoplasmic reticulum, the presence of mitochondria with villous or membranous "cristae", the cilia with the apparently inevitable arrangement of fibrils, and the nuclear membranes separating nucleoplasm from cytoplasm are general features familiar to those studying plant and animal cells. The fungi, too, have mitochondria dispersed within a reticular cytoplasm and there is evidence for a nuclear membrane (1, 15, 27). The cell walls, although not unexpected in the plant kingdom, are extraordinary because of their considerable content of chitin (8).

The summation of common characters given above may seem unnecessarily simplified and undistinguished. Yet a first look, or even a second, at thin sections of bacteria (fixed during active growth) will be enough to allow a general statement that they are very different (Fig. 1). Within the boundary of a distinct cell wall there is an extraordinarily dense cytoplasm which does not display any obvious organelles. Vaguely disposed in the central portion of the cells are discontinuities in the cytoplasmic material. The contents of

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario. The research of the Department has been aided by grants from the National Research Council of Canada and the Atkinson Charitable Foundation. This paper was presented at the Symposium on the Ultrastructure of Cells held as part of the 20th Annual Meeting of the Canadian Physiological Society, Montreal, Quebec, October 20, 1956.

these ramifying spaces have been shown (by light microscopy and staining) to be in part, at least, the chromatin of the bacterial cell. The unique features of bacterial structure lie within this simple description and will be amplified in brief. The reader requiring more information should refer to the various authoritative articles in the recently published symposium *Bacterial Anatomy* (see e.g. 5, 11, 22, 26), which gives excellent description of most of the approaches to bacterial fine structure.

1. *Cell walls*, although they are not confined to the bacteria, can be considered unique in composition. Analyses have been made, particularly by Salton (22), and bacterial cell walls are extraordinarily complex. They contain proteins, carbohydrates, and lipids. Most interesting is that cell walls of Gram-negative bacteria are distinguished from those of Gram-positives by a high concentration of lipid as well as by the absence of certain amino acids. We have found these differences to be reflected in fine structure: Gram-positive bacteria having a dense and almost homogeneous wall; Gram-negative bacteria having the dense–light–dense layering that is so generally found in lipid containing membranes.

2. *The cytoplasm* is, to my mind, quite unique. It seems to consist of closely packed granules ($\sim$ 50 Å) as Bradfield (5) has pointed out. We feel, however, that these are not entirely homogeneous in form. Favorable cuts show some short rodlets, and there may be other distinguishable components (Fig. 2). It would appear that the periphery of the granules scatter electrons more than the center. Experiments involving centrifugal fractionation of bacterial cytoplasm, reviewed recently by Alexander (2), show that most fractions are very heterogeneous. What we can see of the fine structure should not make this result too surprising. There is no evidence yet of membrane systems or of larger organelles, such as a mitochondrial equivalent, and it now seems unlikely that they will be found. Yet one must provide two cautions: the cytoplasmic membranes of bacteria have proved very difficult to demonstrate in sections (12), which indicates that structures can be lost in processing, and there are as yet no helpful studies of photosynthetic bacteria, in which special organelles might be expected (23).

3. *The chromatin structures* of bacteria provide a puzzle. The chromatin areas can be identified (4) but there is no electron microscope evidence that these are separated from cytoplasm by a membrane. In themselves they seem to have characteristics shared only with the blue-green algae (6). In electron micrographs the interpretations must be tentative. My own opinion, which is not shared by all (9, 11, 17, 18), is that the chromatin complex forms a ramifying and interconnecting network for each chromatin body, but details of organization elude us. In any case the general properties, conformation, and behavior of bacterial chromatin cannot really be considered in the same terms as the nucleus of other cells, as Robinow has pointed out (20). The difficulties in preserving the conformation of chromatin structures were discussed at the Symposium but description of these experiments must await

PLATE I

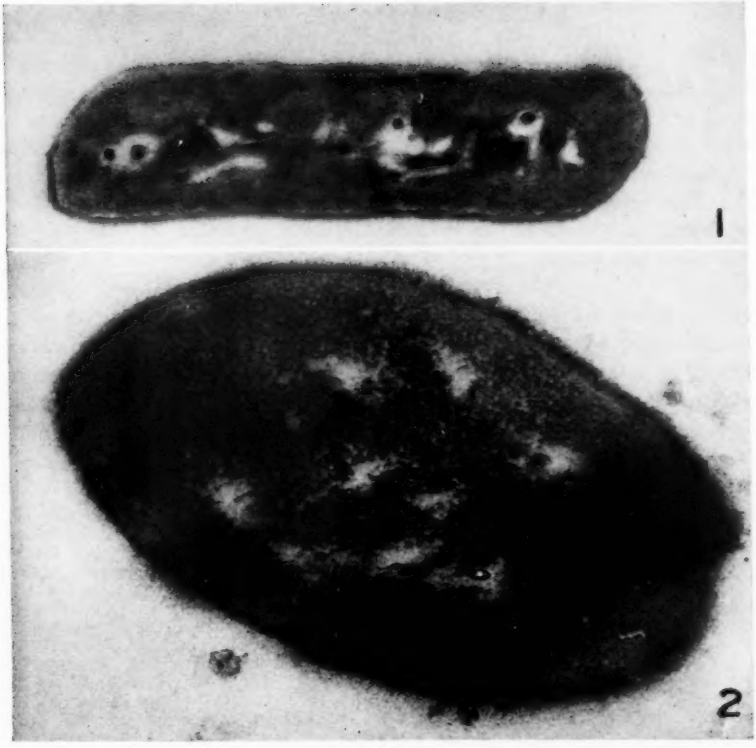

FIG. 1. Longitudinal section of *Bacillus cereus* showing cell wall, dense cytoplasm, and the "vacuoles", containing cords, of the chromatin area. *ca.* 30,000 ×.

FIG. 2. Cross section of *B. cereus* showing the cytoplasmic fine structure and the form and arrangement of the component "granules". The spaces in the cytoplasm are thought to be associated with chromatin and contain material of density and form distinct from cytoplasmic components. *ca.* 86,000×.

separate publication (13). A partial solution to the problem has been attained by controlling the cationic environment of the cells during fixation after the fashion of some of our previous experiments (28).

4. *Flagella*, the hair-like locomotor structures of the bacteria that are motile in fluids, are also unique structures (26). I can say little, however, from our own experience except that they have proved impossible to trace in sectioned material.

In summary it can be said that bacteria show a greater degree of difference in fine structure from the better known plant and animal cells than do the fungi or protozoa. It must be said that the survey within each of the groups is very limited. However, this recalls the difficulties of giving a formal description to a separate kingdom containing the bacteria and the blue-green algae (24, 25), which has long seemed reasonable. It seems possible that this description may be aided materially by studies of the fine structure of these organisms and we await with interest more than a glimpse of the structure of blue-green algae (5) and the photosynthetic bacteria (14).

In the discussion of this paper Dr. Leblond drew attention to the problems of genetic recombination in bacteria. There are undoubted peculiarities in the genetics of bacteria that add to the thesis of the uniqueness of bacteria. The general reader should find some of the general observations of Lederberg (10) both stimulating and in the spirit of this symposium.

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THE PHYSIOLOGICAL ULTRASTRUCTURE OF CELL MEMBRANES[1]

A. S. V. Burgen

Physiological evidence in relation to ultrastructure of cells falls into two classes. Firstly, a study of function may provide evidence for specialized structural arrangements for which anatomical methods may not be available owing to insufficient resolution and lack of specificity. In the past such physiological methods provided valuable information on the nature of the plasma membrane before visualization became possible with the advent of the electron microscope. In the second place even when the ultrastructure of a cell has been studied adequately by electron microscopy, the functional assessment of the significance of the structures depends largely on physiological methods.

The evidence obtained in the study, for instance, of a specific transport process may be clarified finally only when we can take ultrastructure beyond molecular agglomerates down to specific molecular, atomic, and even electron arrangements. The most abundant physiological evidence on ultrastructure relates to the outer or plasma membrane of cells. I will restrict my account to the properties of this structure in animals, although previous speakers have made it clear that the endoplasmic reticulum, and the double layers of mitochondria have an apparently similar structure to the plasma membrane and presumably share some of its properties.

The most fundamental property of the plasma membrane is that it is a phase boundary separating the cytoplasm from the extracellular fluid.

The efficiency of this barrier for ions may be assessed by measuring the electrical conductivity of the membrane in comparison with the aqueous phases on either side of it. Such measurements have been made on many types of cells and indicate a resistance of 1000–10,000 Ω cm.[2] If the plasma membrane is assumed to be of the order of 100 Å thick—this corresponds to a specific resistance of 10^8–10^9 Ω cm. This may be compared with 10^{10} Ω cm. for water-saturated olive oil. Extracellular and intracellular fluids have specific resistances of only 10^2 Ω cm. The membrane thus restricts movement of ions to less than a millionth of that in free solution. A similar conclusion is reached when the permeation of cells by non-electrolytes is considered. For instance the rates of diffusion of a number of substances through the dog's salivary gland were all less than one-millionth of the free diffusion rate (Table I). Even with such small molecules as water and gases a considerable hindrance to diffusion is found.

Any phase boundary that has a high specific resistance compared with surrounding ionic media will act as an electrolytic capacitor. This is revealed

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TABLE I

DIFFUSION OF NON-ELECTROLYTES FROM BLOOD INTO THE SALIVA, CM^2/SEC.

	Restricted diffusion, D′	Free diffusion, D	D/D′
Urea	1.4×10^{-13}	1.7×10^{-5}	1.2×10^{8}
N-ethyl urea	1.2×10^{-12}	1.2×10^{-5}	1.0×10^{7}
Glycerol	1.6×10^{-14}	1.1×10^{-5}	6.6×10^{8}
Mannitol	1.2×10^{-15}	0.84×10^{-5}	7.2×10^{9}
Chloramphenicol	3.7×10^{-13}	0.62×10^{-5}	1.7×10^{7}

in cells by the fact that when current is passed across a membrane the potential reaches a steady level rather slowly and with an exponential time course. From this time course the membrane capacity may be calculated and for most cells it is of the order of 0.5–1 μf./cm.^{2} Now if we know the dielectric constant of the material of the membrane we can estimate the thickness of the phase boundary from the theory of the parallel plate condenser.

In practical units,

$$\text{dielectric thickness (cm.)} = \frac{8.85 \times 10^{-8} \times \text{dielectric constant}}{\text{capacity } (\mu\text{f./cm.}^2)} .$$

The optical and chemical properties of the membrane show that the discontinuity is due to a lipoid layer composed mainly of triglycerides, phosphatides, and steroids. The dielectric constant for such a membrane might be expected to fall in the range between 2.1 for paraffin and 10 for acetic acid. This gives a membrane thickness of 20–90 Å for squid giant axon—thick enough to allow at least for one and probably several lipoid monolayers. But the sartorius muscle of the frog has a capacity of 8 μf./cm.^{2} giving a thickness 2.4–11 Å, barely enough for one monolayer, while crab muscle with a capacity of 40 μf./cm.^{2} the dielectric thickness (0.5–2.2 Å) falls far short of the thickness of one monolayer (Table II). These observations have been interpreted as evidence that because of submicroscopic folding the true membrane area is much greater than that estimated by the microscope. Robertson (9) has found evidence of such folding with the electron microscope. Martin (7) has provided some additional evidence by finding that the conduction velocity in frog muscle is independent of the length to which a fiber is stretched. It seems unwise in our present state of ignorance to conclude

TABLE II

	Membrane capacity, μf./cm.^{2}	Membrane thickness, Å	
		$K=2.1$	$K=10$
Squid giant axon	1	19	88
Frog toe muscle	8	2.4	11
Crab muscle	40	0.5	2.2
Lipoid monolayer		10–20	
Diameter of $-CH_2-$		(2)	
Double layer capacity on mercury surface	20 – 80		

that this can account for all of the "missing thickness". We do not know what the effects of orientation of the membrane lipoid or the effects of variations in thickness on the molecular scale on the dielectric properties of the membrane lipoids may be. For comparison we may note that the capacity on a clean mercury surface during an overvoltage is 20–80 μf./cm.^{2} — just the order of the crab fiber capacity.

Let us turn to some of the more specific properties of the cell. Most permeability studies with non-electrolytes can be understood on simple physical principles. The smallest molecules can penetrate between the elements of the lipoid membrane and the larger molecules can enter only if they are solvated into the membrane. However, in most biological membranes glucose and some other sugars and glycerol behave in an anomalous fashion. Danielli (4) has pointed out that glucose enters the human erythrocyte about one hundred thousand times faster than would be predicted from its oil–water partition coefficient and its molecular radius. Widdas (11) has compared the rate of glucose penetration with that of meso-inositol which has almost identical physicochemical properties — glucose penetrated at least a thousand times faster than inositol. In addition a number of workers have shown that the kinetics of glucose penetration deviate significantly from Fick's law and, further, mutual interferences occur between glucose and other sugars. Finally poisoning the red cell by Hg ions or by phloretin reduces the transport of glucose to a very low level consistent with that expected from its physical properties. These findings can be understood if glucose is transported across the membrane in combination with a specific carrier. This carrier must be a membrane constituent and Widdas has calculated that it probably occupies less than 1% of the erythrocyte surface.

In the erythrocyte no active concentration of glucose occurs and the glucose carrier operates equally well on an inward or outward passage. There is but a short step from this system to the *active* transport against a concentration gradient that occurs in the kidney tubule or intestinal epithelium. In these cases two changes have occurred, the carrier system is now unidirectional, and a source of energy has been coupled in to permit transfer against a free energy gradient. Whether these are independent or mutually dependent steps there is not enough evidence to decide. But the membrane must accommodate additional features to permit transformation of the carrier into an active state at the outer surface and an inactive state at the inner surface.

Special mechanisms operate for many other substances, for instance the cation transport system, commonly called the sodium pump, which maintains the cation segregation between inside and outside the cell. This system expels sodium ions from the cell against the electrochemical gradient, at the same time potassium ions accumulate within the cell; recent evidence suggests that these two processes are much more closely coupled on a common carrier than had been supposed previously. This transport is controlled by both the intracellular sodium and the extracellular potassium.

Quite separate from this pump is the mechanism that allows a transitory increase of sodium permeability across the nerve cell membrane followed by an increase in potassium permeability in generating an active membrane potential. This time separation is essential to the operation and makes it difficult to postulate a single carrier for the two ion fluxes.

A further property of the membrane is that it has what pharmacologists call receptor sites. The evidence that many drugs act on cell surfaces is very good and with some cells we can demonstrate receptors for several drugs on the same cell. For instance intestinal smooth muscle is contracted by acetylcholine, histamine, 5-hydroxytryptamine, pressor hormone, and substance P, all acting at different sites as shown by selective inhibitors and supramaximal addition. The muscle can also be relaxed by adrenaline acting at a further receptor site. Clark (2) showed that for acetylcholine and adrenaline no more than a thousandth of the cell membrane area need be the location of a receptor site.

Perhaps the most dramatic demonstration of a drug receptor is the microstimulation method of Castillo and Katz (1). These workers filled a capillary glass electrode with acetylcholine solution and then ejected it in tiny puffs by applying anodal current pulses. When the electrode was near a muscle end plate each puff of acetylcholine produced a transitory depolarization of the end plate due to an increased permeability to both sodium and potassium. As the microelectrode was brought nearer to the end plate the depolarization became larger until finally when the electrode impaled the end plate so that the acetylcholine was released inside the fiber, no depolarization occurred — the acetylcholine receptor is on the outside of the end plate only.

Further examples of specific properties of cell membranes could be cited, but they all point to a high degree of organization and specialization at the active spots on the membrane. Such a degree of specificity can surely only reside in the protein part of the membrane and this has been sadly neglected by physiologists. There is abundant evidence that the lipoid layer of the membrane is enclosed between two sandwiching layers of protein. A few specific properties of the membrane surfaces are undeniably associated with a very stable protein layer. For instance the outer surface of the erythrocyte has cholinesterase activity. This is so indissolubly connected with the viability of the cell that its turnover is a reliable measure of the life span of the erythrocyte. Similarly Libet has shown that adenosine triphosphatase is a constituent of the inner face of the giant axon membrane and a great many enzymes are known to be insoluble, i.e. associated with cell debris and only solubilized by dissolution of the lipoid part of the membrane. We are certainly in error if we discuss these proteins as *adsorbed:* they are structural proteins forming a vital part of the cell membranes. Taken together with the presence of conducting spots (carriers), which can be regarded as pores in the lipoid of the membrane, this suggests that the inner and outer protein shells may be stabilized by interpenetrating peptide chains. Such peptide chains could account for the fluctuations in membrane density and fenestrations commonly seen in high resolution electron micrographs.

Such peptide chains might well be the basis of specific material conducting systems and indeed a theory based on this supposition is in many ways more reasonable than the current theories which mostly seem to have evolved more from the study of dredges and turnstiles than of feasible chemical systems.

Let us first consider a system that will provide specific conductivity for a single ion in either inward or outward direction without discrimination. If the opposing protein laminae lining a pore had an array of groups as seen in Fig. 1, such that the ion formed a stable combination with two protein groupings, then this arrangement will be specific for ions of particular radius and charge. An ion of the wrong charge will be repelled, and if the ionic radius is too large or too small it will not fit into the matrix of combining groups. Essentially, the arrangement postulated is co-ordination of this ion into a rigid matrix. There is no necessity for the combination to be ionic — for instance hydrogen bonding of glucose hydroxyl groups would do just as well. Once a substance is in the matrix, since all the pairs of combining sites have an equal affinity for the substance, it will tend to jump from site to site in a random manner. Normally the probability of a single molecule being present in the matrix must be less than one or there would be mutual interaction of the influx and outflux. Experimental evidence is usually against such an interaction but Hodgkin and Keynes (6) found that the potassium carrier in the squid axon did show interaction of the fluxes of an order that

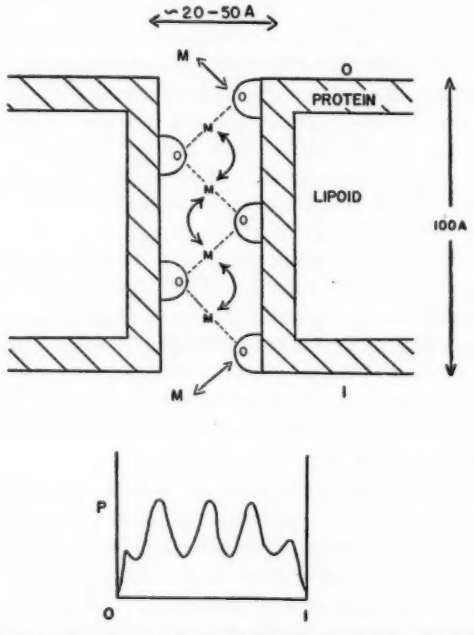

FIG. 1. Above: Arrangement of co-ordinated molecules in parallel sided matrix. Below: Probability of finding a molecule in the matrix.

suggested that two or three potassium ions were normally in the matrix. Before we develop the theory further we must consider whether the membrane thickness can accommodate a large enough peptide chain to make this arrangement possible. If we assume that the membrane is 100 Å thick and that the minimum-sized structure is the size of Pauling, Corey, and Branson's γ-helix with a conducting core 2.5 Å in diameter (8)—this structure has 5.1 residues per turn and a pitch of 5.0 Å—then a 100 Å length would have 102 residues and 20 turns. Larger diameter helices or folded chain arrangements would have a correspondingly larger number of residues. There is therefore room for a medium-sized protein molecule in one of the pores.

Now if we return to the conducting matrix, we can readily make this unidirectional if the spacing between the combining groups shows a steady progression in one direction (Fig. 2). In this case there is a free energy gradient which will force the ion towards that side of the membrane at which the bond lengths are most nearly ideal. This structure will have the important property of segregating ions on one face of the membrane. If some way of detaching the ion from the membrane were available, this would then provide a specific active transport system. Transitory expansion or contraction of the matrix could provide such a means (Fig. 3). On this system one can visualize all the active transport systems of the membrane being supplied with a common energy source provided by the interaction of high energy phosphate compounds with an enzyme on the inner membrane face and undergoing a cycle of contraction and expansion analogous to the contraction – relaxation cycle in muscle that can be induced by fluctuation of ATP concentration. Perhaps the adenosine triphosphatase that Libet found in the membrane of squid axons is this contractile protein. This theory of cyclic matrix change was proposed some years ago by Goldacre (5) on the basis of studies on the motility of *Amoeba*. A common substratum of phosphate energy utilization is appealing because the evidence is so strong that high energy phosphate is important in active transfer and yet there is really no sound evidence that the transported substances are phosphorylated. Indeed Crane and Krane (3) have shown that desoxy sugars are transported nearly as well as glucose by the intestinal mucosa and these have no hydroxyl groups in the 1 and 6 positions and cannot be phosphorylated. Further Taggart (10) has found that no isotopic dilution occurs when *p*-aminohippuric acid labelled on the carboxyl group with ^{18}O is transported by the kidney tubule. This excludes any covalent linkage being formed by the carboxyl group and leaves only ionic or hydrogen linkages as possibilities.

Finally it would be anticipated that the matrix for a relatively large substance such as glucose might have sufficient free space in its core to act as a leak for small ions and water; this accords with the experimental results. Glucose and para-aminohippuric acid in the kidney tubules are transported in one direction only and there is no diffusional back leak, but for potassium in the erythrocyte and iodide in the thyroid follicle a passive component is also found.

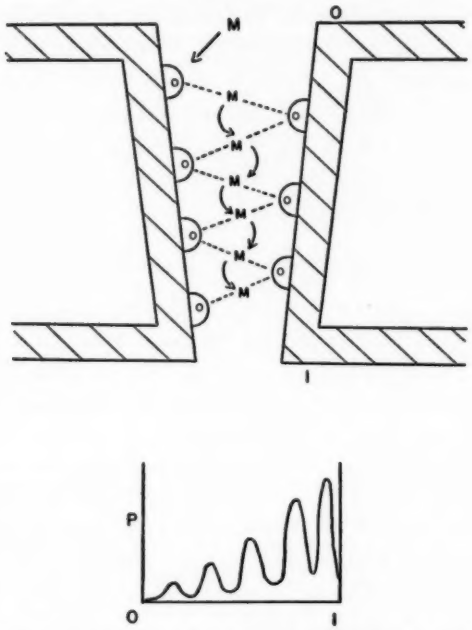

FIG. 2. Above: Arrangement of co-ordinated molecules in a conical matrix.
Below: Probability distribution of molecules.

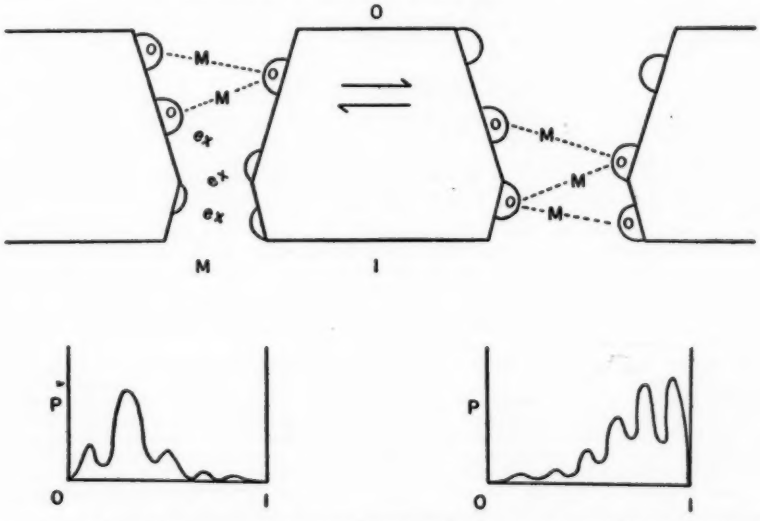

FIG. 3. Above: Arrangement of co-ordinated molecules in an oscillating matrix—left contracted, right expanded.
Below: Probability distribution of molecules.

Addendum in Answer to Questions from the Floor

Dr. Elliott asked about discrimination between sodium and potassium ions. There are quite large differences in diameter between these ions. If the hydrated ions are considered, sodium has a diameter about 40% larger than potassium; with non-hydrated ions this ratio is reversed. Since rather little variation of bond length in a lattice is permissible, these differences should give a marked discrimination between sodium and potassium. It should be noted that this difference in ion diameter is sufficient to give different co-ordination numbers to sodium and potassium and different crystal habits in many of their salts.

In reply to a question by Dr. Kashket, I should mention that, in talking about the external and internal laminae, I was using the term "protein" in its broadest sense to include glyco- and lipo-proteins. With regard to questions by Dr. Nickerson and Dr. Rossiter, the physiological evidence about the nature of the cell membrane has come largely from puncture of cells with fine microelectrodes. When the electrode is inserted an abrupt change of potential occurs as the electrode penetrates the cell membrane but the interior of the cell including the nucleus is isopotential. However, Fatt and Katz occasionally found in crab muscle an intermediate potential immediately after penetration which rapidly gave way to the usual internal potential. Hodgkin has obtained evidence that the membrane around frog muscle may be double in character and enclose a submicroscopic space. Possibly this space might explain some of Dr. Nickerson's results. Finally it does not appear that the interior membranes of the cell restrict the passage of solutes since the diffusibility and mobility of K ions inside the cell are almost identical with those in free solution.

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DISCUSSION: H. B. COLLIER[1]

In commenting on Dr. Burgen's paper I wish to take the opportunity to indulge in some speculation and to suggest possible future avenues of research into the ultrastructure of cell membranes. Since so little is known about their structure in relation to function, speculation seems to be relatively safe. I am chiefly interested in the plasma membrane of the erythrocyte; and for obvious reasons this membrane has been more extensively studied than any other kind.

It is generally agreed that the red-cell membrane is a mosaic of lipide and protein (12) with carbohydrate residues in the lipide (18) and in the mucopolysaccharides of the blood-group substances. Dr. Burgen has referred to the functions of some of the structural elements of the red-cell surface: receptor sites for drugs; enzymes; centers for active transport. It should be possible to elucidate the chemical nature of the receptor sites for drugs and hormones by the use of compounds of known molecular dimensions and charge distributions, as has been done for enzymes by means of inhibitor studies.

The surface enzymes of the erythrocyte have not been very intensively studied in relation to their functions. Even the role of the acetylcholinesterase is still a matter of dispute. Possibly this enzyme is a feature of certain types of cell membranes, but only a vestigial structure in the erythrocyte stroma. The effects of changes in experimental conditions on the composition of the red-cell membrane, and on its enzyme activity, have not been adequately studied. Rose and György (13) showed that tocopherol deficiency in rats made the red cells susceptible to hemolysis by dialuric acid; but we have no information as to whether the red-cell lipides are altered. We[2] have begun a study of the effect of tocopherol deficiency on the enzymes of the red-cell stroma and on the cation gradient of the cell.

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.
[2]With financial support from the Defence Research Board of Canada, Project No. D50-9350-06, Grant No. 9350-06.

Active transport is perhaps the most fascinating problem in connection with physiological ultrastructure. The centers in the cell membrane that are responsible for active transport—whether they are turnstiles, swinging or revolving doors, or charged pores — should be capable of chemical identification. They must form complexes, at least temporarily, with the ions or molecules that they transport across the membrane. The "carriers" for Na^+ and K^+ ions must certainly possess anionic groups. It has been known for some time that the surface of the erythrocyte is negatively charged, yet these anionic groups have not been definitely identified.

Rothstein (14) believes that phosphate and carboxylate are the binding sites for cations of the yeast cell surface. Davies, Haydon, and Rideal (3) concluded from microelectrophoresis studies on *Escherichia coli* that the surface of the cells has the electrophoretic profile of a polysaccharide. The centers for active transport in cell membranes should be identifiable by: binding studies (they probably resemble cation-exchange resins); titration curves; electrophoresis at various pH values.

Melchior (11) has shown that adenosine triphosphate (ATP) does form complexes with Na^+ and K^+. Does it act thus either in the cell membrane or in the interior? Does diphosphoglycerate play a role in complexing these cations?

I am glad that Dr. Burgen revived Goldacre's (6) suggestion of a possible role of contractile protein in cell surfaces. Holtfreter (8) attributed ameboid movements to cation antagonisms on the surface. Kaplan (9) has studied the folding and unfolding of catalase. Flory (5) recently suggested that actomyosin contraction might be attributed to a crystal–liquid equilibrium: perhaps similar changes take place in membrane proteins. Wald (17) has referred to the possibility of energy transfer from molecule to molecule in layered structures, such as cell membranes, by the process of classical resonance.

But I do not think that Dr. Burgen's polypeptide chain structure alone could explain facilitated diffusion or active transport. Simple peptides do not bind glucose or the alkali cations. Granted that the membrane may have a backbone structure of polypeptide: to this must be attached polar groups that are the "carrier" centers. These are probably the anionic groups of nucleotides, phospholipides, and mucoproteins — phosphate, sulphate, and carboxylate.

Is active transport of cations the reverse of muscular contractions? In the latter, a change in cation distribution presumably triggers actomyosin contraction. In the cell membrane, could contraction of charged protein cause movement of the cations in or out? Actomyosin contraction and active transport across membranes may have the following features in common: (1) the role of nucleotide phosphates, as ATP; (2) the participation of cations, such as Na^+ and K^+, Mg^{++} and Ca^{++}; (3) a mechanism involving thiol groups (for muscle see (1) and (10)).

I wish particularly to emphasize a possible very important role of —SH groups in cell membrane permeability and transport. Mercurials cause hemolysis which can be inhibited by thiols, and urethane hemolysis is also inhibited by thiols. Ergothioneine may play a role in the DPNase of the red-cell surface (7). It is well known that the secretory function of the kidney tubule is inhibited by mercurials, and hence —SH groups are probably essential. Bond and Hunt (2), following some work of Davenport, have recently shown that —SH groups are involved in cation secretion by the gastric mucosa. Several Russian workers have suggested that the action of acetylcholine on heart muscle fibers is mediated through thiol groups (cf. 16).

We know that vitamin B_{12} activates protein —SH groups (4). As a matter of pure speculation, does the absorption of B_{12} by the intestinal mucosa require the intermediation of thiols? Ferritin apparently possesses thiol groups. Are they then required for absorption of Fe^{++}?

It is becoming increasingly clear that thiols play a very important physiological role. This may depend on a combination of properties which these groups possess:[3]

oxidation–reduction,
complexing with heavy metals,
ionization to $—S^-$ and H^+,
H-bonding to O or N atoms,
condensation with carbonyl groups.

Much more needs to be learned about the physical chemistry and biochemistry of the thiols. For example, the chemists still have not agreed upon the proper pK value to be assigned to the —SH groups in amino acids such as cysteine and glutathione. There must be some very special physicochemical relationship among protein —SH groups, ATP, and certain cations. These three factors all seem to play a role in muscular contraction, active transport, and the phosphokinase enzymes.

[3]NOTE ADDED IN PROOF. Additional chemical properties of thiols that may be of biochemical significance are: free-radical formation and addition to unsaturated bonds (Koenig, N. H. and Swern, D. J. Am. Chem. Soc. **79**, 362 (1957)); and formation of hydrogen peroxide (Fulton, J. D. and Spooner, D. F. Biochem. J. **63**, 475 (1956)).

As an indirect approach to the problem of transport of Na^+ and K^+ through the red-cell membrane, we[2] are investigating cation interactions in the pyruvic phosphoferase enzyme system of erythrocytes (15). In this system there must be interactions involving the enzyme protein —SH groups, ATP, and K^+ and Mg^{++}. The nature of these interactions may throw light on the mechanism of transport of the cations through the cell membrane, and how the energy from glycolysis is geared to this active transport (presumably through protein via ATP).

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BIOCHEMISTRY OF CELL FRACTIONS[1]

R. J. ROSSITER

In discussing the biochemistry of cell fractions, I am strongly tempted to recall some remarks made by Potter (50) at a recent symposium on a rather similar topic. Potter likened the electron microscopist to some delicate form of alpine life, existing in the peculiar environment of an isolated mountain peak, while the enzyme chemist is on another peak, with no communication between the two. Each flourishes at the particular altitude of his somewhat solitary mountain, where the atmosphere is too rarified for competition from below. Each also cannot survive if he descends to lower altitudes, for here he meets the strong competition from the less specialized forms of life, in this instance the general run of biologists, that inhabit the valley between the peaks.

At the present time the electron microscopist is precluded, for technical reasons, from studying enzymes, whereas the enzyme chemist, for similar operational reasons, is often very far removed from the cell and the conditions of cellular chemistry. Indeed, one feels a certain amount of sympathy with the biologist who was so disrespectful as to describe that ultimate of the enzyme chemist, the crystalline enzyme, as the most highly purified artifact known to biological science.

One of the main tasks confronting the biologist of today is to bridge the valley that separates the peak of the electron microscopist from that of the enzyme chemist. A start has been made with the study of cell fractions, mechanically separated, and limited success has been achieved. Before such studies can be completely successful, however, the metabolic potentialities of each separated cell fraction must be correlated with the ultrastructure of the particles in the fraction, as revealed by the electron microscope.

Mechanical Separation of Cell Constituents

If a small piece of tissue is ground with a pestle and mortar, or fragmented in one of the many types of blendors or homogenizers now available, a suspension of tissue particles is obtained. The suspension is known variously as a dispersion or an homogenate. The latter is a somewhat unfortunate term, since one of the most characteristic properties of such a suspension is that its particles are not homogeneous, either morphologically or chemically. In 1934 Bensley and Hoerr (4) introduced the technique of separating certain of the intracellular constituents, notably mitochondria, by differential centrifugation.* The method was developed further by Claude (10) and subsequently it was improved by Schneider, Hogeboom, and others.

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario. This paper was presented at the Symposium on the Ultrastructure of Cells held as part of the 20th Annual Meeting of the Canadian Physiological Society, Montreal, Quebec, October 20, 1956. The work was supported by grants from the National Cancer Institute and National Research Council of Canada.

Usually the homogenate is separated into four fractions: (*a*) the nuclear fraction, (*b*) the large granule fraction, containing chiefly mitochondria, (*c*) the small granule fraction, sometimes called the microsome fraction, and (*d*) the supernatant or soluble fraction. More recently, some workers have separated a great many more fractions (see below). The most widely used medium for the separation is one that contains sucrose. Hogeboom, Schneider, and Palade (21) used 0.88 *M* sucrose and Schneider (53) used 0.25 *M*, or isotonic, sucrose. The historical development of the subject and the techniques employed in the mechanical separation of cell constituents have been summarized frequently in review articles, two of the more recent being those of de Duve and Berthet (15) and Hogeboom and Schneider (20).

The chemical and biochemical properties of each of the four classical fractions have been determined for a number of different cell types, but by far the most work has been done on mammalian liver cells. Many comprehensive reviews, with extensive tables of the chemical composition, enzyme content, and metabolic potentialities of each of the fractions, are available (15, 20, 22).

Nucleus

The mechanical separation of cell nuclei has been achieved by many workers. The methods used for the obtaining of isolated nuclei have been reviewed by Dounce (14). The chemical composition and function of the nucleus also has been the subject of a great many reviews, two of the more recent being those of Dounce (14) and Mazia (33).

Unlike the other cell fractions, the nucleus contains few enzymes not also present in equally high concentrations elsewhere in the cell. A notable exception is the enzyme system responsible for the formation of diphosphopyridine nucleotide, which appears to be concentrated in the nucleus.

The nucleus contains the genetic apparatus of the cell. This is located in the 'chromatin' of the chromosomes, which are composed chiefly of nucleoprotein. The Feulgen and desoxyribonuclease tests indicate that all of the desoxyribonucleic acid (DNA) of the cell is present in the nucleus (evidence summarized by Swift (59)). This finding is confirmed by direct chemical analyses of separated nuclei. These analyses also show that the nucleus contains significant amounts of ribonucleic acid (RNA).

Currently there is much interest in the DNA of the chromatin. This change of emphasis from the protein of the chromatin to the DNA is due, in part, to the remarkable demonstration by Avery, MacLeod, and McCarty (2) that DNA can produce transformation in microorganisms. Also there is a steadily increasing body of evidence for the view that the DNA is responsible for the genetic determinants within the cell (evidence summarized by Hotchkiss (23)).

When a cell divides, the nuclear DNA, with its specific genetic characteristics, is reproduced. This replication of DNA, by a process not necessarily enzymatic, takes place in the nucleus. On the other hand, reactions that are

under genetic control, i.e. the formation of specific enzyme proteins, take place in the cytoplasm. It is RNA, not DNA, that is associated with this protein synthesis (see below).

Just how the DNA of the gene influences the formation of specific enzymes in the cytoplasm is not known. The RNA of the nucleus (and the nucleolus) has been implicated in this regard. It was suggested by Jeener and Szafarz (25) that RNA is formed in the nucleus and transported to the cytoplasm. Indeed, there is considerable evidence, chiefly from experiments with P^{32}-, C^{14}-, and N^{15}-labelled precursors, that nuclear RNA is more rapidly formed than cytoplasmic RNA. However, it has been claimed that the time relations of the specific activity curves of the nuclear and cytoplasmic RNA are different from those to be expected if there is a direct passage of RNA from the nucleus to the cytoplasm. Another difficulty is that the relative distribution of the bases in cytoplasmic RNA is different from that in nuclear RNA (evidence summarized by de Lamirande, Allard, and Cantero (28)). On the other hand, the experiments of Brachet (6) indicate that, if the nucleus is removed from *Amoeba proteus*, there is considerable loss of cytoplasmic RNA. Also Mazia (33) recently has described experiments with *Amoeba proteus* in which nuclei with labelled RNA were grafted to an enucleated host. Radioautographs would appear to indicate a passage of RNA from the nucleus to the cytoplasm.

In summary, it might be said that there is good evidence for the view that the cell nucleus plays an important role in RNA metabolism. Whether nuclear RNA is a direct precursor of cytoplasmic RNA is not known. The experimental facts are compatible with the hypothesis that some, but not necessarily all, of the cytoplasmic RNA is derived from the nucleus.

Nucleolus

Monty, Litt, Kay, and Dounce (35) have recently summarized the literature concerned with the obtaining of preparations of isolated nucleoli. Nucleoli may be isolated from suspensions of liver cell nuclei ruptured by means of a sonic oscillator. The nucleoli are separated by gravity sedimentations, followed by differential centrifugation.

Chemical examination of these isolated nucleoli indicated that they contain both DNA and RNA. Only a small fraction of the total RNA of the isolated nuclei was present in the nucleoli. This is contrary to the current belief, based on histochemical findings, that nucleoli in general contain much RNA and only small amounts of DNA. It is possible that the relative amounts of RNA and DNA vary greatly with different types of cell.

Because of the cytochemical findings, it has been assumed that the nucleolus plays a prominent part in the RNA metabolism of the nucleus. Monty *et al.* (35) ascribe a much more limited role to the nucleolus. They suggest that the nucleolus "may function primarily in furnishing (directly or indirectly) templates for the synthesis of the particular enzymes that must govern the chemistry of mitosis".

The properties of the nucleus and the nucleolus are summarized in Table I.

TABLE I

NUCLEUS

Fraction	Properties and characteristics		Function
Nuclei	*Most*	DNA DPN-synthesizing enzyme	Genetic apparatus of cell DNA replication
	Some	RNA	Genetic control of enzyme formation (in cytoplasm) ? Forms RNA, which is transported to cytoplasm
Nucleoli	*Some*	DNA RNA	? Concerned with mitosis ? Concerned with RNA synthesis

Mitochondria

Many details of the ultrastructure of mitochondria have recently become known through the use of improved methods of embedding, fixation, and preparation of thin sections by the electron microscopist. The studies of Palade (39, 41) and Sjöstrand (54, 55) indicate that the mitochondria are organized bodies within the cytoplasm, with a characteristic structural pattern. They vary greatly in size from tissue to tissue, from 200 mμ to 500 mμ in diameter and from 300 mμ to up to 5 μ in length. Sedimentation measurements indicate that rat liver mitochondria behave in a fashion similar to that of spherical particles with an effective radius of 220–260 mμ. They are bounded by an outer limiting membrane and an inner membrane connected with incomplete septa, the so-called *cristae mitochondriales*.

Mitochondria isolated from tissue homogenates appear to have the same essential structure (39, 41, 62). Chemically, they contain very little DNA, RNA, or acid phosphatase activity. They contain almost all the cytochrome oxidase and succinic dehydrogenase activity of the cell, some 20–30% of the DPNH-cytochrome *c* reductase and 60–70% of the TPNH-cytochrome *c* reductase activity. They also contain large amounts of many other enzymes, phospholipids, vitamins, and coenzymes.

Isolated mitochondria behave as osmometers. Recent experiments of Watson and Siekevitz (61) appear to indicate that they represent a two-phase system, comprised of a liquid phase containing soluble enzymes in the mitochondrial matrix and a solid phase containing the insoluble or 'particle-bound' enzymes, such as cytochrome oxidase and succinic dehydrogenase. It seems possible that the mitochondrial enzymes are localized in an organized sequence on the internal membrane and its prolongations, the cristae. This would allow for the high rates of oxidation and phosphorylation of which isolated mitochondria are capable, rates which, according to Green (18), are far in excess of those that would be anticipated from random substrate–enzyme collisions in a fluid medium.

Mitochondria can oxidize many substrates completely, including all the intermediates of the tricarboxylic acid cycle and fatty acids. Under favorable conditions the energy derived from these oxidations is stored as 'energy-rich'

phosphate compounds, such as adenosine triphosphate, i.e. the mitochondria are capable of oxidative phosphorylation. All of the enzymes of both the tricarboxylic acid cycle and the electron transport system are present in mitochondria. However, these enzymes are by no means confined to mitochondria. Enzymes such as isocitric dehydrogenase, aconitase, and DPNH-cytochrome *c* reductase are found in much higher concentrations in other cell fractions. In fact, all of the enzymes of the electron transport system, with the exception of cytochrome oxidase, are found outside the mitochondria. Thus many of the reactions usually ascribed to mitochondria may also take place elsewhere in the cell. This applies to the whole of the DPN and TPN electron transport systems, with the exception of the final electron acceptor, cytochrome oxidase, which is confined to the mitochondria.

The mitochondria are usually regarded as intracellular centers capable of providing metabolic energy. They appear free to move about within the cytoplasm of the cell, and examination of living tissue with the phase microscope indicates that they do. However, the mitochondria are concentrated in areas of great activity, e.g. around the I bands of myofibrils and in the axon terminals at interneural and myoneural junctions.

There is also considerable evidence that mitochondria have the ability to transport water and certain electrolytes against chemical gradients. These secretory processes are energy-requiring. Presumably the energy is derived from oxidative phosphorylation.

As to be expected from the role of mitochondria as sources of oxidative energy, many, but by no means all, of the intracellular synthetic processes take place in isolated mitochondrial fractions. Substances such as hippuric acid, citrulline, acetylcholine, and phospholipids, but not protein, are thought to be synthesized within mitochondria.

Lehninger Particles

Mitochondria appear to function as integrated, well co-ordinated, multi-enzyme systems. Disruption of mitochondria by sonic, osmotic, or chemical means yields a number of enzyme-containing, insoluble particles or fragments, probably derived from the inner membrane and cristae. In general, whenever the mitochondrial structure is damaged, for example by the action of snake venoms, as in experiments of Petruscka, Quastel, and Scholefield (47), oxidation proceeds, often at a higher rate, but phosphorylation is inhibited. In the jargon of the biochemist, the phosphorylation is 'uncoupled' from the oxidation.

However, in some circumstances the particles of the damaged mitochondria are still capable of carrying out co-ordinated oxidative processes, to which phosphorylation is coupled. An important example of this was recently reported by Cooper and Lehninger (11). These workers isolated a multi-enzyme complex from digitonin extracts of rat liver mitochondria. The small particles, with a mass of less than 1/2000 of that of the intact mitochondria, are capable of the oxidation of D(−)-β-hydroxybutyrate to acetoacetate, with the transport of electrons to oxygen and the associated phosphorylations.

TABLE II

MITOCHONDRIA

Fraction	Size	Properties and characteristics		Function
Mitochondria	200–500 mμ diam.	*Most*	Cytochrome oxidase Succinic dehydrogenase	Tricarboxylic acid cycle oxidation
	300–5000 mμ long	*Some*	DPNH-cytochrome *c* reductase (20–30%) TPNH-cytochrome *c* reductase (60–70%)	Fat oxidation Electron transport Oxidative phosphorylation Secretory activity *Synthesis of* hippuric acid citrulline acetylcholine phospholipids
	220–260 mμ effective radius (26)	*Very little*	DNA RNA Acid phosphatase	
		Behave as osmometers		
Lehninger particles	1/2000 that of mitochondria	*Some*	Phospholipid Protein	Oxidation of β-hydroxybutyrate Oxidation of succinate Electron transport Oxidative phosphorylation
		Do not behave as osmometers Derived from mitochondria by treatment with digitonin		

The particles contain the whole of the electron transport system, but they cannot oxidize fatty acids or the intermediates of the tricarboxylic acid cycle, with the exception of succinate. Unlike mitochondria, these particles do not behave as osmometers.

The properties of the mitochondria and their constituent Lehninger particles are summarized in Table II.

Endoplasmic Reticulum

Electron microscopic examination of thin sections of most mammalian cells has shown that the cytoplasm is traversed by a complex interconnecting system of tubules and vesicles. This system, called the *endoplasmic reticulum* by Porter (48), has been identified with the basophilic or chromophilic elements of the cytoplasm, a component frequently referred to as the *ergastoplasm* by histologists. Both the technique of ultraviolet microspectrophotometry, as developed by Caspersson (8), and the ribonuclease test (evidence summarized by Swift (59)), indicate that the ergastoplasm is rich in RNA.

Details of the fine structure of the endoplasmic reticulum may be found in the excellent reviews of Palade and Porter (42), Porter (49), and Palade (41). Briefly, the cytoplasm is traversed by an interconnecting network of small tubules, vesicles, and sometimes, larger flat vesicles, called *cisternae* (42). These vesicles are bounded by a limiting membrane, the outer surface of which may be smooth-surfaced or rough-surfaced. The rough surface is caused by small dense particles (10–15 mμ diameter), first described by Palade (40). These so-called *Palade granules* may be attached to the outside of the limiting membrane, but in some instances they are freely distributed in the cytoplasm. The tubules, cisternae, and vesicles, both smooth-surfaced and rough-surfaced, are interconnected. They thus form part of a continuous system and are not discrete unrelated structures.

Where the endoplasmic reticulum reaches the cell surface, the cell membrane appears to be continuous with the membrane lining the vesicles so that the content of the cavities is in direct communication with the extracellular fluid. Where the reticulum reaches the nucleus, the space between the two nuclear membranes (*perinuclear cisterna*) appears to be continuous with the cavities of the reticulum. Watson (60) has shown that the nuclear envelope, i.e. the perinuclear cisterna, is itself penetrated by pores, giving direct access from the nucleoplasm to the cytoplasm.

When homogenates are prepared by the usual means, the endoplasmic reticulum becomes fragmented or, rather, 'pinched off' into smaller components. As has been postulated for some time, these small particles derived from the endoplasmic reticulum are found in the microsome fraction. However, there is some overlap in size between the smaller mitochondria and the larger particles derived from the endoplasmic reticulum. For this reason, homogeneous preparations containing one type of particle only are difficult to prepare. There are many claims in the literature, based on biochemical

and other evidence, that the usual preparations of both mitochondria and microsomes are heterogeneous and contain particles of several types (9, 16, 27, 34, 37, 38, 44).

It is only recently that electron microscope studies of the microsome fraction have been reported (36, 56) and more recently still that electron microscope studies have been carried out along with parallel biochemical observations (26, 31, 43).

Hogeboom and Kuff (19) and later Kuff, Hogeboom, and Dalton (26) reported on the cytoplasmic particulate fractions from rat liver, after centrifugation of liver homogenates in a horizontal preparative rotor of the swinging bucket type. Three families of particles were recognized, having mean effective radii (assuming that the particles are spherical and have a density of 1.20 g./cm.^{3}) of (*a*) 220–260 mμ (mitochondria), (*b*) 120 mμ, and (*c*) 25–55 mμ. As will be seen below, particles (*b*) probably represent the lysosomes of de Duve *et al.* (16) and particles (*c*) probably represent the microsomes, as described by Palade and Siekevitz (43). Although there was some degree of overlap between the different types of particles, the study of Kuff *et al.* (26) is of great value, because it demonstrates that the cytoplasmic particles tend to occur in classes, and that they do not form a continuous spectrum, as first suggested by Chantrenne (9). Although pure preparations were not obtained, the parallel biochemical and electron-microscopic study contributed greatly to many of the conclusions presented below. The particles will be described in order of decreasing size.

Lysosomes

The lysosomes were first recognized by de Duve, Pressman, Gianetto, Wattiaux, and Appelmans (16) because of their distinctive biochemical properties. According to Novikoff, Beaufay, and de Duve (36), they are of the order of 370 mμ in length and, according to Kuff *et al.* (26), they have an effective radius of 120 mμ. The particles, which behave as osmometers, are characterized by possessing almost all the uricase activity and the greatest concentration of acid phosphatase activity (50–60% of the total) within the cell. The particles also appear to contain some RNA (20–30% of the total). They contain no DPNH-cytochrome *c* reductase activity and so may be distinguished both from the mitochondria, which are usually larger, and the microsomes, which are usually smaller. These particles probably represent the acid-phosphatase particles of de Duve *et al.* (16), the more readily sedimented microsomes of Novikoff *et al.* (37), and the large microsomes of Slautterback (56). Both mitochondrial and microsomal preparations are frequently contaminated with lysosomes.

Kuff *et al.* (26) reported that their particles with an effective radius of 120 mμ represent rough-walled vesicles, with attached Palade granules, and they suggested that the particles are derived from the endoplasmic reticulum. However, Novikoff *et al.* (36) consider that the lysosomes represent 'dense bodies', which are scattered throughout the cytoplasm of the parenchymatous

cells of the liver, particularly those along the bile canaliculi. They state that the 'dense bodies' are covered with small electron-dense granules 5–8 mμ in diameter (i.e. smaller than Palade granules).

Little is known of the function of lysosomes. They are rich in hydrolytic enzymes, such as acid phosphatase, β-glucuronidase, cathepsins, ribonuclease, and desoxyribonuclease, and, for this reason, Bennett (3) has suggested that they may be concerned with phagocytosis.

Microsomes

According to Palade and Siekevitz (43), the size of the microsomes varies from 50 to 300 mμ in diameter. Thus they represent a heterogeneous population, the largest overlapping in size with the lysosomes, just as the lysosomes overlap with mitochondria. It is probable that they represent the particles of effective radius 25 or 55 mμ described by Kuff *et al.* (26), for their biochemical properties are similar. The microsomes have the greatest concentration of RNA (50–60% of the total), DPNH-cytochrome *c* reductase activity (50–60% of the total), and glucose-6-phosphatase activity of all the intracellular particles. They contain some protein and phospholipid but, unlike the lysosomes, they possess very little uricase or acid phosphatase activity. Like the lysosomes, they behave as osmometers. They probably represent the less readily sedimented microsomes of Novikoff *et al.* (37).

Palade and Siekevitz (43) state that the microsomes are composed of membrane-bound vesicles, tubules, and cisternae. They are both smooth-surfaced and rough-surfaced, with the latter predominating. The rough-surfaced vesicles have the small dense Palade granules (12–15 mμ diameter) attached to their outer aspect. As stated above, they are thought to be formed by the 'pinching off' of small sections of the endoplasmic reticulum. Their 'particulate' character is probably an artifact of homogenization.

The early experiments of Caspersson (8) and Brachet (5) provided good presumptive evidence that RNA is associated with enzyme synthesis. Experiments with labelled precursors, carried out both *in vivo* and *in vitro*, indicate that the synthesis of enzyme protein takes place in the microsomes. Direct evidence that RNA is involved in protein synthesis is provided by the important experiments of Gale and Folkes (17). These workers showed that RNA is necessary for the incorporation of C^{14}-labelled amino acids into protein and for the net synthesis of enzyme protein by non-viable broken cells obtained by exposing suspensions of *Staphyloccoccus aureus* to supersonic vibration. Spiegelman (57) reported similar findings for suspensions of the so-called protoplasts of *Bacillus megaterium*, obtained by treatment of sensitive cells with lysozyme under hypertonic conditions.

Just how RNA is involved in protein synthesis is still unknown. Many workers are inclined to postulate the presence of a model or template, under the influence of which the building blocks (which may be amino acids or simple peptides) are arranged in the correct order. As pointed out by Brachet (6) in a recent review, "it is tempting to suppose, as many have already done, that it is the RNA that represents the counterpart to the protein".

TABLE III

ENDOPLASMIC RETICULUM

Fraction	Size	Properties and characteristics		Structure	Function
Lysosomes	370 mμ long (36) 120 mμ effective radius (26)	*Most*	Uricase Acid phosphatase (50–60%)	Kuff *et al.* (26) rough-walled vesicles with Palade granules Novikoff *et al.* (36) 'dense bodies', smaller than mitochondria and dotted with granules 5–8 mμ diam.	Bennett (3) ? phagocytosis
		Some	RNA (20–30%)		
		Very little	DPNH-cytochrome *c* reductase		
		Behave as osmometers			
Microsomes	50–300 mμ diam. (43) 25 or 55 mμ effective radius (26)	*Most*	RNA (50–60%) DPNH-cytochrome *c* reductase (50–60%) Glucose-6-phosphate	Palade and Siekevitz (43) membrane-bound vesicles, tubules, and cisternae, with attached Palade granules. Formed by the 'pinching off' of endoplasmic reticulum	Synthesis of enzyme protein
		Some	Protein and phospholipid		
		Very little	Uricase Acid phosphatase		
		Behave as osmometers			
Palade granules	10–15 mμ diam.	*Most*	RNA (80–90% microsomal)	Very dense particles (ribonucleoprotein particles) Derived from microsomes Contribute to basophilia of ergastoplasm	Synthesis of enzyme protein
		Very little	DPNH-cytochrome *c* reductase Protein and phospholipid		
		Do not behave as osmometers			

Palade Granules

These small granules (10–15 mμ diameter) attached to the outer surface of the vesicles of the endoplasmic reticulum have been referred to already. Palade and Siekevitz (43) have separated the microsomes into a soluble membranous portion and a precipitable portion, containing the Palade granules, by treatment with sodium deoxycholate.

These dense granules, when separated, contain most of the RNA of the microsomes (80–90% of the total) and very little phospholipid or DPNH-cytochrome *c* reductase activity. They contain only 20% of the microsomal protein and most of this is nucleoprotein. The granules probably represent the small microsomes described by Slautterback (56), the ribonucleoprotein particles described by Littlefield, Keller, Gross, and Zamecnik (31), and the ultramicrosomes or macromolecules described by others.

The experiments of Littlefield *et al.* (31) indicate that protein synthesis takes place in the Palade granules, which they called ribonucleoprotein particles. Thus while it is the nuclear DNA that in some way determines the enzymatic potentiality of a cell, it is the RNA of the Palade granules, attached to the vesicles of the endoplasmic reticulum, that is intimately concerned with the replication of enzyme protein. DNA is not necessary for this process, for Brachet and Chantrenne (7) showed that enucleation produced no immediate effect in the cytoplasmic protein synthesis of *Acetabularia mediterranea*, and the experiments of Spiegelman (57) and Littlefield *et al.* (31), referred to above, would appear to rule out a direct effect of DNA. Concerning the method of replication of enzyme protein, some of the more recent observations on the mechanism of formation of induced enzymes in microorganisms, recently reviewed by Spiegelman and Campbell (58), may be of great importance.

The properties of the lysosomes, microsomes, and Palade granules are summarized in Table III.

The Cell Sap

The supernatant remaining after the removal of the particulate fractions is said to be derived from the cell sap. This fraction, which contains small amounts of protein and phospholipid, is fairly rich in enzymes. However, the possibility that these enzymes were originally present in other particulate fractions and were split off during the process of preparation should be borne in mind.

The supernatant contains all the enzymes necessary for the synthesis of glycogen and all the enzymes necessary for glycolysis. It is widely believed that glycolysis is confined to the cell sap. However, many of the glycolytic enzymes are found in quite high concentrations in other cell fractions. LePage and Schneider (30) studied anaerobic glycolysis in isotonic sucrose homogenates of rabbit liver. Although the supernatant was found to have the greatest glycolytic ability, this activity was considerably enhanced by the addition of other fractions.

With brain tissue, water homogenates are capable of a much more rapid rate of glycolysis than homogenates prepared in isotonic sucrose or isotonic potassium chloride (Table IV). When the hypotonic homogenate is separated into a supernatant and a particulate fraction, only a small part of the glycolytic activity is present in the separated supernatant and even less is in the particles (Table V). Not until the two fractions are recombined is maximal glycolysis restored. Presumably the particles contain an enzyme that is rate-limiting in glycolysis. One possibility is hexokinase. Crane and Sols (13) reported that 90% of brain hexokinase is present in the mitochondria.

It can thus be seen that while the cell sap contains all the glycolytic enzymes, it does not follow that all of the steps of glycolysis necessarily take place outside the intracellular particulates.

TABLE IV

EFFECT OF HOMOGENIZING MEDIUM ON THE ANAEROBIC GLYCOLYSIS OF HOMOGENATES OF RAT BRAIN

Medium	μM. lactate/60 mg. wet wt./hr.	% of that with water homogenate
Hypotonic (distilled water)	66.8	100
Isotonic (sucrose)	13.2	20
Isotonic (KCl)	16.8	25

TABLE V

FRACTIONATION OF WATER HOMOGENATE OF RAT BRAIN

Fraction	Anaerobic glycolysis (μM. lactate/100 mg. wet wt. original tissue/hr.)	% of that with whole homogenate
Whole homogenate	68.9	100
Particles	5.8	8
Supernatant	17.1	24
Particles + supernatant	74.6	108

Organization of Metabolic Processes

From the foregoing discussion it is apparent that many metabolic sequences are isolated in certain discrete structural units within the cell. Just how important this structural organization is to the control of metabolic processes is not quite clear at the present time. There are, however, certain indications that such a spatial and structural organization may be of much greater significance in the control of intracellular metabolism than has been suspected hitherto. One such indication is the finding that when metabolic control is lost, as in the neoplastic cell, there is an associated derangement of intracellular ultrastructure. For instance, Howatson and Ham (24) have shown that there are changes in the ergastoplasmic structures of the cells of liver tumors and that there is a reduction in the number of mitochondria, thus confirming the previous report of Allard, de Lamirande, and Cantero (1).

Another indication of the importance of structural organization becomes evident when the various factors that can control or alter cellular metabolism are considered. In general, intracellular metabolic processes can be controlled or altered by (*a*) vitamins, vitamin deficiencies, or antivitamins, (*b*) toxic substances, natural or synthetic, (*c*) drugs and antibiotics, and (*d*) hormones, produced by the body itself. To date, biochemists have achieved some success in describing, at the enzyme or coenzyme level, the action of many of the vitamins and antivitamins (e.g. thiamine, niacin, riboflavin, pyridoxine, pantothenic acid, folic acid, *p*-aminobenzoic acid, vitamin A), the action of some of the toxic substances (e.g. heavy metals, cyanide, carbon monoxide, fluoracetate), and that of a few drugs (e.g. anticholinesterases, some antibiotics, 2,3-dimercaptopropanol). In these instances, the postulates of enzyme chemistry, together with the concept of the 'biochemical lesion', as developed by Peters (45), and the concept of substrate competition, as developed by Quastel and Wooldridge (52) and Woods (63), have sufficed for an acceptable description (see also Quastel (51)).

However, there are many instances where such classical concepts have proved totally inadequate to explain the methods whereby metabolic processes are controlled or altered. These areas of conspicuous non-success include the action of some of the vitamins (vitamin D and vitamin E, in particular), the action of many naturally occurring toxic substances (especially the toxic alkaloids), the action of most drugs (e.g. the analgesics, the hypnotics, the narcotics, the analeptics, the ataractics, the antipyretics) and, most notable of all, the mechanism of action of the hormones.

With the hormones, some effects on metabolic processes have been observed in tissue preparations after the hormone has been administered either *in vivo* or *in vitro* (e.g. insulin, adrenaline, glucagon, thyroid hormone). However, it is significant that the most convincing effects have been obtained with slice preparations, where ultrastructure is complete, or with mitochondrial preparations, where some considerable degree of ultrastucture is preserved. With isolated enzyme systems the effects that have been described are far from convincing. As Cori (12) has recently written "although phosphorylase has been implicated in the metabolic action of epinephrine, hexokinase in that of insulin, and oxidative phosphorylation in that of thyroxine, none of these systems is sufficiently well understood to make one confident that the results obtained so far explain the actions of these hormones".

The idea that many hormones exert their effect through the structural elements of the cell—the *cytoskeleton*—was recently advanced by Peters (46). It is suggested that the effect of a hormone is not on an enzyme or enzyme system, but on an enzyme-structure complex. The recent observations of Lehninger and colleagues (29) on the effect of the thyroid hormone on mitochondrial structure are of interest in this regard.

The intracellular ultrastructure may be equally important in the action of many drugs. For example, Magee, Berry, and Rossiter (32) showed that the

TABLE VI

EFFECT OF CHLORPROMAZINE AND AZACYCLONOL ON THE LABELLING OF PHOSPHATIDE IN BRAIN PREPARATIONS

Preparation	Specific activity (cts./min./μg. P)			Specific activity (cts./min./μg. P)		
	Control	Chlorpromazine (10^{-4} *M*)	% increase	Control	Azacyclonol (10^{-2} *M*)	% increase
Slices (guinea pig brain)	39	69	79	50	77	54
Mitochondria (rat brain)	151	190	26	131	144	10
Water homogenate (rat brain)	101	107	6	116	120	3

tranquillizing drugs, chlorpromazine* and azacyclonol†, increased the labelling of phospholipid from inorganic P^{32} in brain slices, without affecting the labelling of the acid-soluble phosphates. Table VI shows that with each of these drugs the effect is readily obtained with slices, it is relatively much less with mitochondria, and it is virtually non-existent with hypotonic homogenate preparations.

Thus with some drugs, as with the hormones, metabolism is modified, only if a considerable degree of cellular structure remains. It would appear that there are two levels of organization within the cell at which metabolic processes may be controlled:

(*a*) *The enzyme level.*—This classical method of control is the more primitive. It is the method whereby vitamins, antimetabolites, and a whole range of enzyme inhibitors affect metabolism. At this level, substrate competition, enzyme competition, and the pure 'biochemical lesion' are operative.

(*b*) *The enzyme-structure level.*—This method of control is more sophisticated. The ultrastructure of the cell, or at least that of the intracellular organelles, must be maintained. It is the level at which many drugs, and the hormones, influence cellular metabolism. The method of control probably arose relatively late in the evolutionary process, at a time when the enzymatic and structural pattern of the cell, as well as its nutriture, were well developed. The control is probably mediated by way of the organized enzyme complexes associated with the intracellular organelles. When the cellular ultrastructure is destroyed, control is no longer possible. At the present time the secrets of the enzyme-structure level of organization lie inaccessible in the valley between the peak occupied by the electron microscopist and the summit occupied by the enzyme chemist. It was stated at the outset that one method of bringing the inhabitants of the two peaks together is the study of mechanically separated cell fractions. The present state of knowledge in this field has been reviewed. It seems reasonable to assume that with future progress the bridge between the two peaks will slowly take shape. With the building of the bridge, piece by piece, there will be a further understanding of the processes whereby

*Largactil (3-chloro-10(3′-dimethylaminopropyl)phenothiazine hydrochloride), Poulenc Limited, Montreal, Canada.

†Frenquel (α(4-piperidyl)benzhydrol hydrochloride), William S. Merrell, Company, Cincinatti, Ohio.

intracellular metabolism is controlled. Ultimately, the bridge will connect not only the peak of the electron microscopist with that of the enzyme chemist, but it will spread out in many directions to other, more distant, summits. These might well include the still very isolated peaks of the pharmacologist. the toxicologist, the endocrinologist, and finally, the physiologist.

Addendum in Answer to a Question from the Floor

In answer to a question by Dr. C. Allard (Montreal Cancer Institute), in relation with RNA synthesis, there is no doubt but that both nucleus and cytoplasm can synthesize RNA; we are all agreed on that. For a long time biochemists have been very suspicious of the idea that RNA might pass from the nucleus to the cytoplasm. However, I think that the reason for discarding this view was not a very good one. It was based on two pieces of experimental evidence: (*a*) the study of specific activity – time relations and (*b*) the study of the constituent bases of nuclear and cytoplasmic RNA, such as that carried out here in Montreal by de Lamirande, Allard, and Cantero. These studies indicate that, in the main, the nuclear RNA and cytoplasmic RNA are two different things, with different metabolic origins, but they do not disprove the hypothesis that *some* RNA may reach the cytoplasm from the nucleus. Such RNA would not necessarily have the same constituent bases as either the pooled nuclear RNA or the pooled cytoplasmic RNA. Everyone wants very much to find something that goes from the nucleus to the cytoplasm, because of the various effects that the nucleus exerts on the formation of enzymes within the cytoplasm. We now believe that this protein synthesis takes place in the Palade granules. These granules are rich in RNA, so it would be very nice if the something that goes from nucleus to cytoplasm turned out to be RNA. For a while it was generally thought that the passage of RNA from nucleus to cytoplasm was a "dead duck", but more recent work, particularly that with the enucleated amoeba, must now be considered. For the present, we have no alternative but to leave the question open. In my slides I inserted interrogation marks and I thought I had hedged enough with 'mays' and 'mights' in my presentation. It may happen that some RNA is found in the nucleus and, as suggested by Dr. Allard, it is degraded to quite small units before it passes to the cytoplasm. It is difficult to determine where RNA becomes "not-RNA", i.e. polynucleotide. Nuclear RNA might be degraded to small molecules, pass through the holes in the nuclear membrane by processes similar to those described by Dr. Burgen, recombine to form large molecules, which produce the known specific effects on protein synthesis in the cytoplasm. It is true that the nucleus, by reason of its genetic determinants, does influence protein synthesis in the cytoplasm. The problem still is: how does it do it?

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DISCUSSION: LOWELL E. HOKIN[1]

In addition to those cellular components mentioned by Dr. Rossiter as having been isolated one might also add two highly specialized structures, the Golgi bodies and the zymogen granules. Schneider and Kuff (5) have isolated Golgi bodies by centrifugation of epididymal homogenates layered over a sucrose density gradient. Under the phase contrast microscope the isolated Golgi bodies had a similar appearance to those seen in unfixed epithelial cells of the epididymis. No measurable DNA was found in the Golgi bodies, but they contained 20% of the cellular RNA and 25% of the cellular phospholipid. In fact, the Golgi bodies contained a considerably higher concentration of RNA and phospholipid than any other cellular fraction isolated. The Golgi bodies were also very rich in alkaline phosphatase, a fact which was previously suggested by histochemical studies.

Zymogen granules have been isolated in our laboratory (1). The starting material was the pancreas of dogs; pancreatic glands from pigeons were tried but did not give a satisfactory separation of the zymogen granules from the other fractions. The zymogen granules from dog pancreas homogenates sedimented as a white layer at 1000 $\times$ g in 0.25 M sucrose. When suspended in 0.25 M sucrose the zymogen granules appeared under the microscope as highly refractile spherical bodies ranging from about 0.5 to 1.5 microns in diameter.

The zymogen granule fraction contained only traces of RNA and very small amounts of phospholipid. The RNA was probably present as a contaminant, and this may also apply to most of the phospholipid. The very low concentrations of RNA and phospholipid in the zymogen granule fraction indicate that this fraction was fairly pure, since all of the other fractions were very much richer in both RNA and phospholipid. Of the various cellular fractions isolated the zymogen granule fraction contained the highest concentrations of amylase, lipase, and protease, as one might expect from the function of the zymogen granules as storage sites for the digestive enzymes.

The zymogen granules were stable for long periods in isotonic sucrose at pH 5–6, but if the pH was raised above 6 they dissolved. Solubilization was complete at pH 7.2 or above. This solubilization of the zymogen granules at alkaline pH is consistent with the fact that one never sees zymogen granules in the alkaline secretion of the pancreas. The optimum stability of the zymogen granules at pH 5–6 suggests that the pH of the pancreatic acinar cell, at least in the vicinity of the zymogen granules, may lie in this region. Amylase activity was determined in all of the fractions, and considerable activity was found in the nuclear fraction, the mitochondrial fraction, and the soluble fraction. Since amylase is an enzyme specially elaborated for digestive purposes the distribution of amylase in the various particulate fractions should be a good measure of the distribution of zymogen granules in these fractions. Microscopic examination revealed that the nuclear fraction was grossly contaminated with zymogen granules. The mitochondrial fraction was also contaminated. The amylase activity in the soluble fraction was presumably due to the release of amylase into solution on homogenization. This solubilization was 95% if the zymogen granules were suspended in water. These data demonstrate the degree of contamination of different fractions by a single cytological component.

Other studies which we have carried out also show marked overlapping of fractions. When protein secretion is stimulated in slices of pancreas or salivary glands the turnover of phosphoryl units in certain phospholipids is markedly stimulated (3). In an attempt to find out in which cellular component the phospholipid effect occurs we have homogenized secreting and non-secreting slices of pancreas after incubation with P^{32} and separated the various fractions by differential centrifugation. The phospholipid effect was greatest in the microsome fraction, but significant effects occurred in all of the other fractions. These studies show that if the phospholipid effect is occurring in only one structure of the cell this structure is being distributed to an appreciable extent in other fractions. Fragments of the intracellular membranes or the endoplasmic reticulum of liver homogenates sediment mainly in the microsome fraction, as Dr. Rossiter has mentioned. Electron microscope studies have shown that the membranous structures of the endoplasmic reticulum in the acinar cells of the pancreas are longer and more extensive than in the parenchymal cells of the liver. It is therefore possible that in pancreas homogenates the fragments of the endoplasmic reticulum range more widely in size and distribute themselves in many fractions in addition to the microsome fraction. I mention these data to emphasize the caution which must be exercised in concluding that a fraction of a tissue which sediments at a similar centrifugal force to that of a liver fraction contains the same cytological components. In tissue fractionation work each tissue appears to be a law unto itself.

Dr. Rossiter has discussed the importance of cellular structure in the action of hormones and drugs, and he has cited as an example the smaller stimulations by chlorpromazine and azacyclonol of P^{32} incorporation into the phospholipids of brain homogenates as compared to slices. Acetylcholine (with eserine) stimulates P^{32} incorporation into the phospholipids of

[1]Contribution from the Department of Pharmacology, McGill University, Montreal, Quebec.

guinea pig brain cortex slices about 100% (2); the stimulation in homogenates or cytoplasmic particulate fractions prepared in isotonic sucrose is on the average about 25–30% (4). The phospholipid effect in pancreas and in salivary glands, which I have already referred to, is completely abolished by homogenization.

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DISCUSSION: CLAUDE ALLARD[1]

Dr. Rossiter has made a comprehensive and thorough survey of the biochemistry of cell fractions. Every investigator in this special field, as well as in biochemistry in general, will benefit from his excellent review. Unfortunately, owing to lack of time, only a few points can be discussed.

Dr. Rossiter has mentioned the few liver enzymes which actually can be demonstrated in pure nuclei isolated by differential centrifugation. To this small list indeed, we may also add deoxyribonuclease. Recent observations in our laboratory (3) showed that pure liver nuclei do contain deoxyribonuclease activity. However, this enzyme could be demonstrated only in the presence of Ca ions at a molar concentration of 0.5×10^{-3}. This finding apparently solves the problem of having DNAase present solely in the cytoplasm and DNA in the nuclei.

Many metabolic sequences have been shown to be isolated in discrete structural units of the cell and Dr. Rossiter has pointed out that this arrangement may suggest biochemical links between various morphological parts of the cell. The localization of the total cytochrome oxidase activity in mitochondria illustrated such a link between these particles and the cell sap in the electron transport system. Dr. Rossiter mentioned hexokinase as a possible link between glycolysis in the cell sap and in the morphological units.

In connection with this, I wish to mention here another series of reactions where enzymes, although mostly found in the soluble phase of the cell, can nevertheless be connected with organized cytoplasmic structures. The purine-metabolizing enzymes so far studied (guanosine and inosine phosphorylases, guanase, adenosine deaminase, and xanthine oxidase) were found to be concentrated solely in the cell sap. The exception is uricase, the enzyme which is terminal in that sequence. Uricase is known to be localized exclusively in cytoplasmic elements which are intermediate between mitochondria and microsomes (4).

Dr. Rossiter has pointed out in the last part of this lecture that metabolic processes might be controlled at two levels (1) a primitive one: the enzyme level, and (2) a more complex one: the enzyme-structure level. I would like to comment on this by discussing briefly the behavior of several enzymes and other liver components in conditions where the liver cytoplasm is markedly affected.

With the collaboration of Dr. de Lamirande, we had the opportunity of studying the effect of fasting on the levels and intracellular distribution of various liver enzyme activities (2). Ribonucleic acid and nitrogen content as well as the number of mitochondria were also determined. In these experiments, nuclear counts were done so that all data could be expressed per average liver cell. Briefly, we observed that 7 days' fasting caused a 50% loss of cytoplasmic mass. This 50% loss of cytoplasmic material was paralleled by a 50% loss of mitochondria, ribonucleic acid, nitrogen, "ATPase", uricase, inosine phosphorylase, and cathepsin enzymatic activities. Thus, these components apparently changed in amount or in activity in proportion to the mass of cytoplasm. Therefore, their concentration per unit mass of cytoplasm remained unchanged in starved rats. However, a loss of 90% in xanthine oxidase activity was observed in the livers of fasted rats as compared to the fed controls. On the other hand, several liver enzymes were maintained in starved animals at the same level of activity per cell as those of fed animals. These were acid and alkaline ribonucleases, glutamic dehydrogenase, acid phosphatase, guanase (2), and glucose-6-phosphatase (8).

These data show that the localization of a component in a particular morphological unit of the cell cannot indicate *a priori* the behavior of the enzyme. Prolonged fasting caused a loss of mitochondria and RNA per cell. However, a mitochondrial enzyme (glutamic dehydrogenase) and a microsomal enzyme (glucose-6-phosphatase) were maintained per cell. On the other hand, two enzymes which are localized in the same part of the cell (xanthine oxidase and guanase) and involved in the same metabolic process behaved quite differently. Finally, acid phosphatase maintained its activity per cell whereas two other lysosomal enzymes, cathepsin and uricase, decreased to 50% of the level found in the average liver cell of fed animals.

Experiments done with hepatoma transplants present problems of special interest. In this liver tumor, glucose-6-phosphatase (7), glutamic dehydrogenase (1), acid phosphatase (1),

[1]Contribution from the Research Laboratories, Montreal Cancer Institute, Notre Dame Hospital, Montreal, Quebec.

and uricase (5) as well as xanthine oxidase (5) activities could not be demonstrated or were extremely low. The absence of these enzymes in hepatoma cells is of interest in view of the marked morphological changes observed in these tumor cells under the electron microscope (6).

These data illustrate, I think, the delicate control mechanism which must exist at all levels of organization of the cell—both morphological and molecular.

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Notes to Contributors

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